FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Medicago truncatula as a platform for Molecular Farming: a study of the subcellular localization of a human recombinant protein

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Dissertation presented at Faculdade de Ciências e Tecnologia of Universidade Nova de Lisboa to obtain the Degree of Master in Biotecnology

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LISBOA

2012

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Work performed at the Plant Cell Biology Lab at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, under the supervision of Dr Rita Abranches and co-supervision of Dr Ana Sofia Pires.

Electron microscopy analysis performed at Cellular Biology Lab at Instituto de Conservación y Mejora de la Agrodeversidad Valenciana, Universidad Politécnica de Valéncia, under the supervision of Dr José Maria Simarro.

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.” (William Bragg Sr., 1915)

Acknowledgments

I would like to devote this space to thank the people that helped me in the achievement of my master thesis.

First, I would like to thank my parents, Angelina and Mário, and my sister, Bárbara, who always manifested their confidence in me and in my work. Thank you for your huge support along all these student years. Thank you for all the encouraging words and advice in all those difficult and happy moments. Thank you for your showing your happiness so effusively when even the smallest goal was achieved. But most of all, thank you for all the love.

To my friends who kept up with me along all these years. Thank you Eliane, Joana, Sérgio, Spark, Nicas, Rita, Marçal, Sofia, Castelo, Flores, Cláudia, Bifes, Fábio, Rui. For all the support and for all the good times experienced so far (and so many more to come).

I want to express my huge gratitude to the Plant Cell Biology Lab, which welcomed me so well. Especially to my thesis supervisor, Rita Abranches, a very special thanks for giving me the opportunity to develop my very interesting master's thesis, for always challenging me, for teaching, for caring and for contributing to my development as a scientist. A BIG thank you.
To my thesis co-supervisor, Sofia Pires, thank you, for all the support along this past year, for your affection, availability and confidence in my work. Thank you for all the conversations shared. It was a pleasure.

To Sílvia Tavares, thank you for your empathy and interest in my work. Thank you for showing yourself always available for anything I needed and for all the pleasant conversations.

To Cláudia Nogueira, my lab partner and friend, thank you. For all the work ideas shared, for your help and patience. But mainly for all the moments and laughs shared.

I also want to thank to the people that welcomed me in my internship in Valencia. A special thanks to José Maria Seguí Simarro, for your availability, interest and teaching. To Patricia Corral Martinez, my lab guide and friend during the internship in Valencia; thank you for all your teaching and for all the moments shared that made me not want to leave.

To Helena Silva, thank you for your curiosity and for giving me the opportunity to get a clue of what teaching is.

Thank you all.

Rita Santos

Resumo

O uso de plantas transgénicas para a produção de proteínas recombinantes com interesse comercial e farmacêutico oferece várias vantagens quando comparado com os sistemas tradicionais. A utilização de sistemas de planta inteira tem potencial para estudos de tráfego de proteínas e o sistema de culturas em suspensão combina as vantagens da planta com os benefícios de produzir uma proteína recombinante em culturas celulares.

Nesta tese de mestrado, foram utilizadas plantas *Medicago truncatula* expressando uma proteína humana do tipo lipocalina, prostaglandina D_2 sintase (L-PGDS). Este estudo apresenta a primeira análise da produção desta proteína nesta planta específica e o primeiro estudo do tráfego subcelular desta proteína em plantas. Foram analisados dois sistemas de produção: sistemas de planta inteira e culturas em suspensão. A presença da L-PGDS foi estudada, através de Western Blotting e microscopia de fluorescência e electrónica, em três diferentes órgãos da planta (folha, raiz e semente) e nas células em suspensão e meio celular.

A proteína L-PGDS mostrou acumular-se em diferentes locais e padrões consoante o tipo de célula em que estava a ser produzida. Trabalhos recentes têm demonstrado que a especialização funcional das células vegetais nos órgãos de armazenamento pode influenciar o tráfego subcelular das proteínas recombinantes, e assim, o destino da proteína pode ser diferente entre sementes e folhas da mesma planta transformada. Foi também demonstrado que a espécie de planta em estudo, onde a proteína recombinante é produzida, pode alterar os parâmetros de tráfego.

A *Medicago truncatula* é, assim, um sistema promissor para a produção de proteínas recombinantes. Os resultados iniciais obtidos neste estudo poderão contribuir para estudos futuros e para o desenvolvimento de *Molecular Farming*.

Palavras-chave: *Molecular Farming*, *Medicago truncatula*, L-PGDS, proteína recombinante, microscopia de fluorescência, microscopia electrónica.

Abstract

The use of transgenic plants for the production of recombinant proteins with commercial and pharmaceutical value offers several advantages when compared to the standard systems. Whole plant systems have potential for studies of the recombinant protein trafficking and suspension cell cultures combine the advantages of plants with the benefits of protein production by cell cultures.

In this master thesis work, *Medicago truncatula* plants expressing a lipocalin-type human prostaglandin D_2 synthase (L-PGDS) were used and the protein trafficking was studied. This study was the first analysis of the production of this protein in this specific plant and the first study of the subcellular trafficking of this protein in plants. Two production systems were analyzed: whole-plant systems and suspension cell cultures. The presence of the L-PGDS was studied in three different plant organs (leaf, root and seed) and in the cells and cell medium, by Western Blotting and fluorescence and electron microscopy.

The L-PGDS protein appeared to accumulate in different places and patterns depending on which type of cell it is being produced. Recent work has shown that functional specialization of plant cells in storage organs can influence the subcellular trafficking of recombinant proteins, so the protein subcellular fate could be different between seeds and leaves of the same transformed plant. It has also been demonstrated that the plant species where the recombinant protein is produced, can alter the trafficking parameters.

Medicago truncatula is, thus, a promising system for the production of recombinant proteins. The initial results obtained in this study could contribute to future studies and development in Molecular Farming.

Keywords: Molecular Farming, *Medicago truncatula*, L-PGDS, recombinant protein, fluorescence microscopy, electron microscopy

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Abbreviations

2,4-D – 2,4-Dichlorophenoxyacetic Acid

Asn – Asparagine

BCIP – 5-Bromo-4-Chloro-3-Indolyl Phosphate

BDMA – Benzyldimethylamine

BSA – Bovine Serum Albumine

bp – Base Pairs

cDNA – Complementary DNA

CHS – Chitin synthase gene

CSF – Cerebrospinal fluid

Da – Dalton

DAPI – 4',6-diamidino-2-phenylindole

DDSA – Dodecenyl Succinic Anhydride

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

EGTA - Ethylene glycol-bis(2-aminoethylether)-N,N,N ',N'-tetraacetic acid

ER – Endoplasmic Reticulum

EM – Electron Microscopy

Fuc – Fucose

Gal – Galactose

Glc - Glucose

NAc – N-Acetylglucosamine

Glu – Glutaraldeheyde

GSH – Glutathione

L-PGDS - Prostaglandin D_2 Synthase Lipocalin type

H-PGDS - Prostaglandin D_2 Synthase Hematopoietic type

HDEL – Histidine-Aspartic Acid-Glutamic Acid-Leucine

KDEL – Lysine-Aspartic Acid-Glutamic Acid-Leucine

Man – Mannose

MS – Murashige & Skoog

MTSB – Microtubule Stabilizing Buffer

MW – Molecular Weight

NBT – Nitro-Blue Tetrazolium

NeuAc – Neuraminic Acid

NMA – Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride

PBS – Phosphate Buffer Saline

PBS-T – Phosphate Buffer Saline with Tween 20

PGD_2 – Prostaglandin D_2

PGH_2 – Prostaglandin H_2

PGDS – Prostaglandin D_2 Synthase

PIPES – Piperazine-N,N′-bis(2-ethanesulfonic Acid)

RNA – Ribonucleic acid

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Ser – Serine

TEMED – Tetramethylethylenediamine

Thr – Threonine

TSP – Total Soluble Protein

UV – Ultra Violet

Xyl - Xylose

WT – Wild Type

1 Introduction

1.1 Molecular Farming

Plants have been important to humans and human survival, from fruits and natural dyes to pharmaceuticals and materials such as cotton and sugar. In fact, human population has been choosing the better fruits, which look better and are not poisonous, for centuries. This process was an earlier version of genetic engineering, once the most interesting features of plants were chosen over others. Therefore, current plants do not look like the ones common centuries ago.

Nowadays, the pharmaceutical field is important, once it is imperious to produce new and more effective compounds in a safer and inexpensive way, and plants offer several metabolites with many different therapeutic effects. Plants naturally develop these compounds, so they can protect themselves against pathogens and predators. There are compounds with several functions such as wound-healing, anti-inflammatory, antimicrobial or psychoactive properties (Boehm, 2007).

In the 1980s, several improvements were achieved in the field of plant biotechnology, caused by the development of strategies and techniques that enabled the insertion of exogenous DNA in plants by infection with *Agrobacterium tumefaciens*, allowing heterologous protein production (Fraley *et al.*, 1983). Hiatt and colleagues first reported the production of recombinant antibodies in plants in 1989. They demonstrated that functional antibodies could be produced and assembled in transgenic tobacco leaves (Hiatt *et al.*, 1989). With these advances, large-scale production of proteins was achieved and named “Molecular Farming”, as it allows the production of important and commercially valuable proteins in plants (Fischer & Emans, 2000; Schillberg *et al.*, 2003).

Molecular Farming presents an alternative to other existing processes, since it offers several advantages such as economic benefits (growing a plant is more economical in material and personnel than settling a fermentation or a bioreactor system), safety (plant production reduces health risks from pathogen contamination, for it does not contain human or animal pathogens) and storage benefits (it is easier to store a small plant seed, which as a plant will produce the desired protein, and seeds remain stable at ambient temperatures for months or even years) (Giddings, 2001; Schillberg *et al.*, 2003). Furthermore, plants can also perform post-translational modifications needed to obtain a correct protein biological activity. For all these reasons and advantages plants are now seen as a new source of molecular medicines and other products of commercial interest.

Plants are expected to achieve a higher economic benefit than other production systems because they have greater capacities of storage flexibility and scalability and the production costs are lower, when compared with most of the systems used nowadays. Thus, once the investment is made, the return will be higher.

Along with consecutive developments in this area of study, several methods to produce recombinant proteins in plants appeared in plant sciences. There are two main strategies to express a recombinant protein in a plant: transient expression and stably transformed plants. The first strategy allows the production of the recombinant protein without the permanent transformation of the plant cell genome and, therefore, has the advantage of not resulting in transgenic plants, which carry several ecologic and regulatory concerns, and has proven to be successful for rapid, small and medium-scale production. It can be used to test the function of an expression construct before proceeding to transform the plant cell genome permanently and eventually to a large-scale production. The latter one requires a longer time for generation and characterization of produced lines, as well as the generation of enough seed material (Boehm, 2007; Abranches *et al.*, 2005; Schillberg *et al.*, 2003).

The most frequent strategy is stable nuclear transformation, which requires transferring foreign genes into plant cells and then to incorporate them within the host nuclear genome, by infection with *Agrobacterium tumefaciens* or by particle bombardment. This way of stably transforming a plant is advantageous, since the seeds produced by this plant will also be transformed and, due to the high resistance of seeds and their capability of being stored without suffering degradation, it becomes easier to propagate the transformed plant (Abranches *et al.*, 2005).

However, Molecular Farming systems also present some disadvantages. The most notorious is the difference in the glycosylation patterns between plants and mammals. This characteristic is of such importance, once it glycosylation patterns are one cause of immune responses in humans that research on this area is urgent so glycosylation pathways can be more clearly understood and needed pathway modifications can be applied in the future. Several strategies have been developed to solve this problem, such as directing the protein to a certain organelle in the cell or accumulating the protein in a specific organ (Boehm, 2007). This will be discussed later on.

1.2 Molecular Farming Products

For decades, several compounds have been produced such as vaccines, antibodies, biopharmaceutical proteins and industrial proteins. In 1986, Barta and collaborators made the first breakthrough in this research field when they achieved the expression of a gene encoding the human growth hormone in plants (Barta *et al.*, 1986). Hiatt and colleagues also brought novelty in this field, once they assessed plants as a cost-effective bioreactor to produce antibodies of different types (Hiatt *et al.*, 1989; Hiatt *et al.*, 1990).

1.2.1 Vaccines

Charles Arntzen first foresaw plant vaccines or edible vaccines in the early 1990s, and since then this topic became an important field of Molecular Farming research (Mason *et al.*, 1992). Plant vaccines are a novel system of delivery because the vaccine itself is produced by the plant and accumulated in the fruits or the seeds. This progress simplifies the delivery and the administration of the vaccine, besides reducing the production costs. The delivery is simplified since sophisticated refrigeration systems are not needed, allowing an easier transportation and storage. Production costs are lower and there is no need of purification steps since the fruit or seed is orally administrated. For this purpose the choice of a plant species like banana or some other fruit is important, since the plant has to have an edible part that can be eaten by humans or animals without cooking or any kind of processing. The major concern with edible vaccines is the degradation of protein components in the stomach and gut before they promote an immune response. In 1998, Mason and colleagues suggested an alternative processing when they tested a possible vaccine against enterotoxigenic strains of *Escherichia coli*. The vaccines consisted in subunit B of the heat-labile toxin (Lt-B), expressed in corn. They suggested that a typical 1 mg dose of Lt-B would be delivered in the embryo fraction (decreasing the dose volume) or in a cooked whole corn snack (increasing palatability). The final conclusion was that neither treatment degraded the antigen and its biological function remained stable (Mason *et al.*, 1998). Thus, plant vaccines are a promising field of research since it seems a safer and cost-effective way to achieve mass immunization compared to other systems (Boehm, 2007; Daniell *et al.*, 2001).

1.2.2 Antibodies

Antibodies are an important tool for the diagnosis and treatment of several diseases and thus a promising field of research. Recombinant antibodies with the same biological activity than antibodies produced by other systems have shown to reduce immunogenic reactions. In addition, functional recombinant antibodies have been successfully produced in plants and stored in plant leaves and seeds. This shows the potential of long-term storage of protein and the reliability of this production system (Kaiser, 2008; Giddings, 2001; Boehm, 2007).

1.2.3 Biopharmaceutical proteins

One of the greatest breakthroughs in recent years was the production of a Gaucher's disease therapeutical protein. In 2007, Shaaltiel and colleagues discovered a way of producing, at a large scale, human recombinant glucocerebrosidase in carrot cell cultures (Shaaltiel *et al.*, 2007). Nowadays, Protalix, an Israeli company, is producing this recombinant protein in plastic bag reactors with transgenic carrot cell cultures. This way of producing this compound reduces the production costs compared with mammalian expressing systems (Kaiser, 2008).

Despite all advantages of plant systems, biopharmaceutical protein production gives rise to some concerns such as transgenic crops affecting food supply by contamination of the non-transgenic crops. There is also a limitation in the level of recombinant proteins produced in transgenic plants, since the common values obtained are less than 1% of the total soluble protein needed for commercial feasibility. Another concern is the possibility of human immunoreactions after administration. This problem can be solved by humanizing the plant, as will be explained later. So, plant-derived pharmaceuticals have to meet high standards of safety and performance for production to become cost-effective (Boehm, 2007; Daniell *et al.*, 2001).

1.2.4 Industrial Proteins

Industrial proteins are also an important product of Molecular Farming since it is a cheaper way of producing these compounds. In 1997, Chong and colleagues have achieved the expression of recombinant human milk protein β-casein in transgenic potato plants (Chong *et al.*, 1997) and in 2002, Nandi and collaborators expressed human lactoferrin in transgenic rice seeds (Nandi *et al.*, 2002). These two proteins are examples of industrial applications of proteins produced in plants, in this case for infant formulas and baby foods. Other kinds of proteins are also being produced by this system such as amylases, phytases and hydrolases (see Table 1.2.4.1). Molecular Farming will be improved when high levels of expression can be achieved, as this affects the production costs and, thus, the investment. In any case, this way of producing industrial proteins has much more room for improvement than other systems already established (Horn *et al.*, 2004).

Table 1.2.4.1 – Biopharmaceuticals for human health produced in transgenic plants (Daniell *et al*., 2001).

Potential application or indication	Plant host	Protein	Expression levels	Refs[a]
Human proteins				
Anticoagulant	Tobacco	Human protein C	<0.01% TSP[b]	36
Thrombin inhibitor	Canola (*Brassica napus*)	Human hirudin	0.30% seed protein	36
Neutropenia	Tobacco	Human granulocyte-macrophage colony-stimulating factor	Not reported	50
Growth hormone	Tobacco	Human somatotropin, chloroplast	7.00% TSP	42
	Tobacco	Nuclear expression	<0.01% TSP	42
Anemia	Tobacco	Human erythropoietin	<0.01 TSP	34
Antihyperanalgesic by opiate activity	*Arabidopsis*	Human enkephalins	0.10% seed protein	34
Wound repair and control of cell proliferation	Tobacco	Human epidermal growth	<0.01% TSP	36
Hepatitis C and B treatment	Rice, turnip (*Brassica rapa*)	Human interferon-α	Not reported	36
	Tobacco	Human interferon β	<0.01% fresh weight	34
Liver cirrhosis, burns, surgery	Tobacco	Human serum albumin	0.02% TSP	34
Blood substitute	Tobacco	Human hemoglobin α, β	0.05% seed protein	36
Collagen	Tobacco	Human homotrimeric collagen	<0.01% fresh weight	51
Cystic fibrosis, liver disease and hemorrhage	Rice	Human α-1-antitrypsin	Not reported	50
Trypsin inhibitor for transplantation surgery	Maize	Human aprotinin	Not reported	50
Antimicrobial	Potato	Human lactoferrin	0.10% TSP	52
Non-human proteins				
Hypertension	Tobacco, tomato	Angiotensin-converting enzyme	Not reported	50
HIV therapies	*Nicotiana bethamiana*	α-Tricosanthin from TMV-U1 Subgenomic coat protein	2.00% TSP	50
Gaucher's disease	Tobacco	Glucocerebrosidase	1.00–10.00% TSP	36

[a]Because of space limitation, reviews that cite original citations are provided.
[b]TSP, total soluble protein.

1.3 Molecular Farming Biosafety

Most recombinant proteins produced in mammals or bacteria present several contaminants, which are absent in plants, a feature that makes plant systems particularly suitable for production of therapeutical proteins, vaccines and other important recombinant proteins. Thus, plant systems are particularly suitable for production of therapeutical proteins, vaccines and other important recombinant proteins. However, plants present also some disadvantages such as the presence of contaminants like pesticides and transgene pollution, causing environmental safety concerns that must be addressed. The use of transgenic plants in Molecular Farming raises the concern of contamination of non-transformed crops or of the environment while harvesting, transport and processing takes place. Another matter, not less important, is that all recombinant proteins produced in plants to be administered in humans must be compared to their native counterparts and undergo several safety tests. Transference of transgenes to non-transgenic plants can be prevented by elimination of marker genes or superfluous sequences or by physical or genetic containment, removing the source of pollution (Commandeur *et al.*, 2003).

1.4 The Choice of the Farming System

The choice of the farming system requires some tests and several prerequisites to ensure that the chosen system presents optimal conditions for the production of the specific protein and its final application. One important concern is to ensure and establish high yields of the recombinant protein to make the production system cost-effective. Another concern is to guarantee the stability of gene expression over generations, ensuring the continuous production of the recombinant protein without the need of another transformation. Protein accumulation must be the next step to analyze, since protein accumulation in certain cell compartments has shown to promote higher yields (Conrad & Fiedler, 1998). So, the choice of the production system must be decided on a case-by-case basis, depending on the protein to be produced, the planned product characteristics and its final use. Thus, for a system to be effective it has to meet good product yield functionality and good product homogeneity, as well as the post-translational modifications. For this reason, the success of the production system depends on a clear understanding of cellular pathways and mechanisms for each plant species and for tissue-specific factors that will affect protein expression, and therefore its quality and accumulation (Abranches *et al.*, 2005).

1.5 The Plant Medicago truncatula

Medicago truncatula belongs to the Fabaceae family, is original from the Mediterranean basin and is found in a wide range of habitats. It is also used as stock feed in leys or permanent pastures of the subtropical areas of Australia and the USA (Djemel *et al.*, 2005; Maureira-Butler *et al.*, 2008).

Medicago truncatula was established as a model plant for the legume family (Cook *et al.*, 1997; Cook, 1999) and it shows several conserved genome regions with alfalfa and pea, it is a diploid autogamous species, it has a short genome and life cycle, and is easy to manipulate and transform in laboratory (Djemel *et al.*, 2005). The phylogenetic distance to economically important crops is crucial in the choice of this plant by many researchers and by funding agencies, since it allows comparative genetic studies within the legume family. Besides, high levels of expression of a model recombinant protein have been observed in transgenic *Medicago truncatula* (Abranches *et al.*, 2005). Such high levels of expression allow transgenic plants to be cost-efficient to maintain *in vitro* and easy to propagate under sterile conditions. The legume family is one of the most important groups of plants, since it provides several products necessary worldwide, as food for animals and humans, oil and available nitrogen (Abranches *et al.*, 2005; Ané *et al.*, 2008).

1.6 Post-translational Modifications and Subcellular Deposition

Most mammalian proteins have to undergo several post-translational modifications to be functional including proteolytic cleavage, addition of glycan chains, phosphorylation, hydroxylation or acetylation (Abranches *et al.*, 2005; Gomord & Faye, 2004).

The first methods used to produce recombinant proteins, especially for pharmaceutical purposes were bacterial fermentation (*Escherichia coli*), yeast cell cultures (*Saccharomyces cerevisiae*, *Pichia pastoris*) and mammalian cell cultures. Bacterial systems are known for their simplicity in manipulation and scaling-up although they also present setbacks such as not being able to fold proteins correctly, since the glycosylation process is absent, and the occurrence of protein accumulation and aggregation. Yeasts systems, on the other hand, can carry out glycosylation, even though the N-glycan chains are very different between yeasts and mammals. In addition, this kind of system presents a high rate of product loss. As for mammalian cell cultures, they are very complex and sophisticated because they mimic the protein production in the human body, reproducing the post-translational modifications almost perfectly. However, these cultures present high production costs due to the expensive infrastructure and material. They are also very difficult to scale-up because the reactor volume limits the process. Besides, mammalian systems present the risk of contamination with pathogenic organisms, such as viruses and prions, as well as oncogenic DNA sequences (Schillberg *et al.*, 2003).

Protein glycosylation is the process that consists in covalently linking a sugar moiety, or glycan, to a protein, thus being an essential process of the post-translational modifications. Glycans can take various forms and can be linked to proteins in several ways. Therefore, plant glycoprotein modifications are of special interest as plants are a possible system for the production of compounds like pharmaceuticals and feed additives that are usually glycosylated. Since N-glycosylation is so essential for a good protein functionality and stability, the analysis of this process is of extreme importance when dealing with recombinant protein production (Abranches *et al.*, 2005).

One of the main advantages of transgenic plants over the production systems based on yeast or bacteria is their ability to accomplish most of the post-translational modifications needed for the bioactivity of recombinant proteins. Other production systems present several disadvantages. For instance, bacteria cannot carry out important post-translational modifications such as signal peptide cleavage, protein folding or disulfide bond formation. In contrast, plants perform the majority of the post-translational modifications required for a protein to be biologically active. The attachment of carbohydrates to the protein backbone affects several physicochemical properties, such as resistance to thermal denaturation, protection from proteolytic degradation, half-life, solubility and even alters crucial biological functions (Gomord & Faye, 2004).

In plants, N-glycosylation occurs preferentially to O-glycosylation (when the sugar is attached to the hydroxyl end of Thr or Ser residues in the peptide backbone) and starts in the endoplasmic reticulum (ER). Here, a co-translational transfer of an oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, to a specific asparagine residue in the N-glycosylation sequence Asn-X-Ser occurs (being X any amino acid except proline). After being transferred to the nascent protein and while the new glycoprotein is transported along the secretory pathway, the oligosaccharide N-linked to Asn undergoes maturation steps like the removal of glucose and mannose residues to form high mannose-type glycans and the addition of new sugar residues in the ER and Golgi apparatus, thus generating complex-type N-glycans. There are four different types of N-glycans that can be generated due to the substitutions of the common core ($Man_3GlcNAc_2$): high-mannose type, complex type, paucimannosidic type and hybrid type (Figure 1.6.1) (Gomord & Faye, 2004; Saint-Jore-Dupas *et al.*, 2007; Rayon *et al.*, 1998).

High-mannose type glycans from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ are originated from the limited trimming of the glucose and mannose residues present in the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$. Complex type N-glycans result from the processing in the Golgi apparatus of high-mannose type N-glycans by the action of specific glycosidases and glycosyltransferases. This type of glycans is characterized by the presence of α (1,3) fucose residue linked to the proximal N-acetyl glucosamine and/or by the presence of β (1,2) xylose residue linked to β-mannose residues of the core. It is also characterized by having one β (1,2) N-acetylglucosamine residue linked to α-mannose units. N-glycans containing α (1,4) fucose and β (1,3) galactose have been identified as being linked to N-acetylglucosamine terminal units.

These modifications result in sequences of Gal β1-3(Fuc α1-4)GlcNAc known as Lewis[a]. These structures have been previously found on cell surface glycoconjugates of several primates (Watkins, 1995 and Dupuy *et al*., 2002) and in secreted glycoproteins of *Xenopus tropicalis* (Guérardel *et al*., 2003). In contrast, Lewis[a] structures are widespread among plants (Fitchette *et al.*, 1999; Wilson *et al.*, 2001). The third type of N-glycans, paucimannosidic type, are oligosaccharides found in all plants that have only one α (1,3) fucose residue and/or one β (1,2) xylose residue linked, respectively, to the proximal GlcNAc and to β-mannose residues of the common core $Man_3GlcNAc_2$ or to the restricted core $Man_2GlcNAc_2$. This type of N-glycans arises from the elimination of the terminal antenna resulting in a truncated form of complex type N-glycans and can be considered as typical vacuolar-type N-glycans. The last type of N-glycans, the hybrid type, results from processing the single branch of the α (1,3) mannose of the $Man_5GlcNAc_2$ intermediate, leading to oligosaccharides with α (1,3) fucose and/or β (1,2) xylose residues linked to $GlcNAcMan_5GlcNAc_2$ (Rayon *et al.*, 1998).

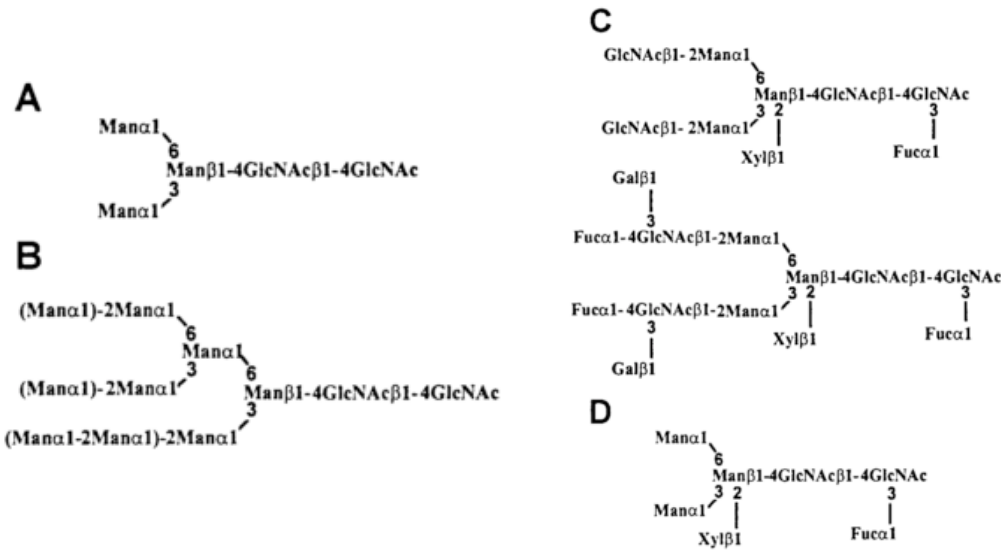

Figure 1.6.1 – Structure of plant N-glycans. A – common core of N-linked glycans; B – high-mannose-type N-glycans; C – complex type N-glycans; D – paucimannosidic type N-glycans (Rayon *et al.*, 1998).

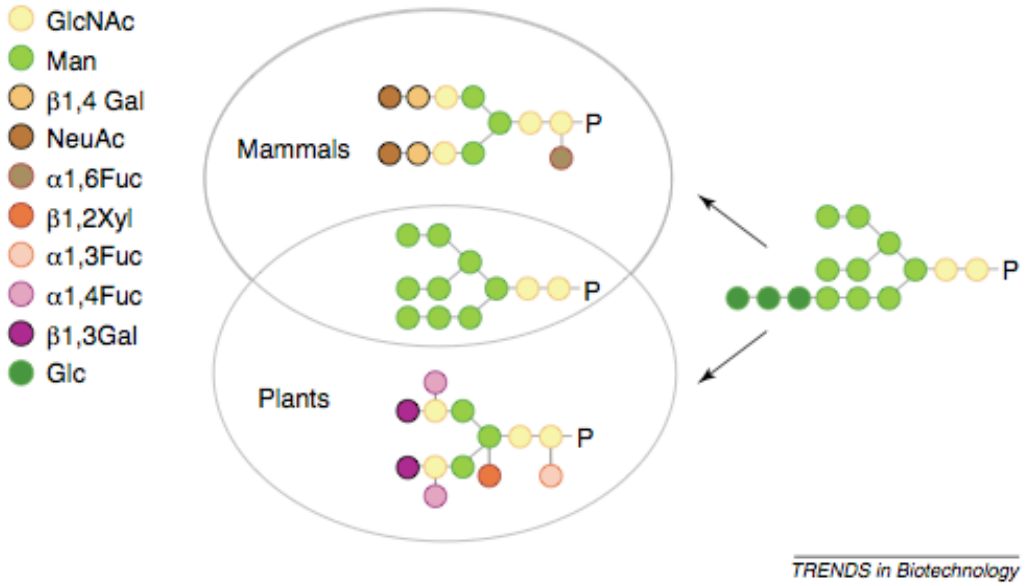

Figure 1.6.2 – Differences between plants and mammalian complex N-glycans (Gomord *et al.*, 2005).

Furthermore, N-glycan structures from plants differ from those found in mammalian (Figure 1.6.2). As the high-mannose type N-glycans contain five to nine mannose residues and have the same structure in plants and mammalian glycoproteins, on the other hand, complex type N-glycans are structurally different between these expression systems. In plants, β-mannose is substituted by a bisecting β (1,2) xylose that is not found in mammalian N-glycans, and the proximal N-actylglucosamine of the core is substituted by an α (1,3) (Lerouge *et al.*, 1998). When concerning the terminal galactose and the sialic acid, plants and mammals also differ in this aspect, since plants lack the β (1,4) galactose and terminal α (2,6) N-acetyl neuraminic acid (a sialic acid) residues, that are often found in mammalians (Sturm *et al.*, 1987).

1.6.1 Glycosylation Optimization and Humanization

Since glycosylation patterns of a recombinant protein produced in plant are often not totally the same as the original mammalian protein, strategies of humanization of the plants have been developed. Folding, stability and post-translational modifications of a given recombinant protein will be affected by targeting the protein to certain cell compartment, changing its accumulation pattern. Strategies to target the recombinant protein to a desired location are therefore an option that allows control of the final product.

The addition of an N-terminal signal peptide will cause the co-translational import of the protein to the ER. Further addition of the tetrapeptide KDEL or HDEL (lysine/histidine, aspartic acid, glutamic acid, leucine) will retain the protein in the ER (Franken *et al.*, 1994). KDEL/HDEL causes the protein, in the secretory pathway, to be retrieved from the Golgi apparatus and transported back to the ER, so it is not lost in the continuous flow of proteins to the plasma

membrane. The absence of any target signal will promote the accumulation of the recombinant protein in the cell apoplast (Abranches *et al.*, 2008).

Another possible strategy to achieve this objective is engineering glycosylation in plants, performing a knock-out of some specific genes, inhibiting Golgi glycosyltransferases plant specific, as they are responsible for N-glycan maturation (Schillberg *et al.*, 2003; Gomord *et al.*, 2010; Saint-Jore-Dupas *et al.*, 2007). Strasser and colleagues (2004) have generated mutants of the *Arabidopsis thaliana* plant with N-glycans of the complex type lacking a β (1,2)-linked xylose and a core α (1,3)-linked fucose (Strasser *et al.*, 2004). Interference RNA also proved to be a valuable tool to knock-down β(1,2) xylosyltransferase and α(1,3) fucosyltransferase in *Nicotiana benthamiana* (which transfers a L-fucose sugar from a GDP-fucose to the GlcNAc core) (Strasser *et al.*, 2008). Furthermore, it is possible to express heterologous glycosyltransferases to add sugar residues common in mammalian proteins. These enzymes can be targeted to a specific subcellular localization improving the knock-in strategies. It is also possible to glyco-engineer a plant to add sialic acid in a galactose residue, characteristic of human glycoprotein (Misaki *et al.,* 2006; Paccalet *et al.*, 2007; Castilho *et al.,* 2010). The removal of the Lewis^a epitope has to be taken into account because it will cause an immunogenic reaction in mammals (Gomord *et al.*, 2010).

Efforts in N-glycosylation engineering are concentrated on the combination of strategies that inhibit the biosynthesis of plant glyco-epitopes with complementation strategies, with the ultimate goal of obtaining fully humanized and sialylated N-glycans. Rapid progress towards humanization in plants is currently turning plant-engineered N-glycosylation to an advantage for this promising system.

1.6.2 Localization *vs.* Glycosylation

Since the enzymes that are responsible for the addition and modification of glycans are compartmentalized, the structure of N-glycans reflects the pathway, trafficking and deposition of the protein within the plant cell. Therefore, the glycosylation pattern of one protein can be controlled by trafficking and deposition. Thus, the final glycosylation pattern exhibited by a protein will reveal its pathway from its synthesis until its final destination. Several strategies can be applied to obtain a protein with the desired characteristics as explained in chapter 1.6.1.

In the eukaryotic cells, for the polypeptides to be destined to the secretory pathway the cDNA construct has to contain a signal peptide that facilitates the transport to the ER. Then, proteins can be secreted to the cellular surface or to the endomembrane system. The subcellular fate and the pathway followed by the glycoprotein through the endomembrane system will determine the N-glycan final structure, since its core is modified by several glycosidases and glycosyltransferases (Rayon *et al.*, 1998).

It has been suggested that glycan modification could be influenced by several factors including plant species or even by the organ where the protein accumulates. So, if protein

glycosylation is dependent on its intracellular localization, differences in subcellular deposition can also occur between tissues, species and physiological stages (Abranches *et al.*, 2005).

Further evidence showed that the fate of a determined recombinant protein is intrinsically linked to the tissue where it is produced. Drakakaki and colleagues (2006) studied a transgenic rice plant transformed with phytase from *Aspergillus niger* and observed that the enzyme was secreted to the apoplast in the leaves and accumulated in the ER and in protein storage vacuoles in the endosperm cells. Thus, the phytase subcellular fate differed according to the type of tissue where it was being expressed (Drakakaki *et al.*, 2006).

Abranches and colleagues (2008) studied the same recombinant protein in another model plant, *Medicago truncatula*. They transformed the *Medicago* plant in two different ways: one cDNA construct with a signal peptide directing the protein to the secretory pathway (SP-phy) and one cDNA construct also with the signal peptide but with an additional KDEL signal for ER retention (KDEL-phy). In the SP-phy plant, phytase accumulated in the apoplast as expected. In KDEL-phy plant, the enzyme was detected in protein storage vacuoles and in the ER, as expected for a protein accumulating in the ER. This suggests that the final fate of a recombinant protein can be controlled by the addition of signals interpreted by the cell as an instruction to the subcellular targeting; which proves that each plant species behaves differently even thought the protein is the same (Abranches *et al*., 2008). Hence, this suggests that the functional specialization of plant organs influences the subcellular fate of a given protein. Arcalis and colleagues (2004) observed that recombinant phytase and human serum albumin produced in wheat seeds showed unexpected deposition patterns in the post-endoplasmic reticulum compartments suggesting that cereal seeds possess a more complex protein-sorting machinery than other plant tissues (Arcalis *et al.*, 2004).

Several intracellular locations have been tested for recombinant protein production, such as cytosol, ER, apoplastic space, vacuole and chloroplast (Boehm, 2007). ER gives rise to the highest yields of biologically active protein due to the fact that in this organelle proteins are more protected from degradation. In contrast, cytosolic expression leads to low levels of protein yield since proteases are present in the cytosol. Apoplastic expression also gives rise to good yields, since proteins also undergo modifications in ER and Golgi apparatus that mature and stabilize proteins (Boehm, 2007).

1.7 Plant based platforms

Leafy plants, like alfalfa and tobacco, are the ones that show a greater biomass yield, as they can be harvested several times a year. Nevertheless, leaf production has a disadvantage since these plants have to be kept in cold storage facilities or processed immediately at the harvesting site, summing additional costs. In contrast, seed crops, like cereals produce less biomass but provide excellent storage properties. When a transgene is expressed constitutively in the whole plant, the recombinant protein has to be extracted right after harvesting and protein

purification has to be performed, otherwise the protein will start to degrade as the plant organs start to decompose, except for the seed that can preserve its state for a good amount of time (Twyman *et al.*, 2003). These steps represent additional costs in the final process, being simpler to target the recombinant protein to a storage organ like the seed, for instance.

However, there are other strategies to obtain a good final product, like the fusion of the recombinant protein with oleosins (proteins within the oil bodies). If the recombinant protein is produced as a protein fused with oleosin (with a protease recognition site inserted between them), the final protein will accumulate in the oil bodies membrane, being easily purified by centrifugation. The oil droplets can be mixed with an aqueous medium and the oleosin cleaved from the protein of interest. A new centrifugation will result in two separation phases, where the aqueous medium will contain the recombinant protein (Hsieh & Huang, 2007). Another important technique is the use of an inducible promoter, which is also a good strategy to increase recombinant protein expression. A unique ethanol-inducible process was proposed by Werner and colleagues (2011). This is achieved by inducing, with ethanol, the release of viral RNA replicon leading to RNA amplification and high-level protein production (Werner *et al.*, 2011).

Another strategy is the application of a unique transmembrane, as well as cytoplasmic tail sequences used as anchors to deliver recombinant proteins via distinct vesicular transport pathways, thus increasing the expression in vacuoles (Jiang & Sun, 2002). Strategies like the incorporation of an intron into the 5' untranslated region of a gene, the choice of a terminator or even the coupling of a gene promoter with its cognate terminator can significantly influence the level of transgene expression (Dugdale *et al.*, 1998; Mitsuhara *et al.*, 1996; Outchkourov *et al.*, 2003).

1.8 Plant Cell Cultures

Plant cell cultures are an *in vitro* system that can be used for recombinant protein production under carefully controlled conditions (Figure 1.8.1). Haberlandt foresaw the theory for this system in 1902, when he established the plant cell totipotency concept (the ability of a single cell to divide and give rise to all the differentiated cells of an organism), being the cell the primary unit of all living organisms (Haberlandt, 1902). His work did not produce successful results but impelled future developments.

Plant cell cultures have the potential to synthesize a variety of useful, low molecular weight molecules, present rapid production cycles and high levels of containment. This strategy of producing recombinant proteins uses cheaper medium components compared to the ones used in mammalian cell cultures and lacks human pathogenic particles. Notwithstanding, this methodology cannot compete with open field cultivation since production costs are higher, but still promise to be more cost-effective than mammalian cell cultures (Boehm, 2007). On the other hand, whole plant systems present several disadvantages when compared to plant cell

cultures: they present a longer development time, variations in product yield and quality, along with the difficulty in applying good manufacturing practices in the early steps of production (Boehm, 2007). In addition, in whole plant systems the possibility of contaminations with agrochemicals is present, cultivation conditions are variable, as well as the impact of pests and diseases. Additionally, this production system is intrinsically safe, since it is not infected with human pathogens or presents endotoxins. Moreover, maintaining this system is simple, because it uses a synthetic medium. This expression system is independent of climate, soil quality, season, day length and weather (Hellwig *et al.*, 2004).

Plant cell cultures can be initiated with a *callus* which is an undifferentiated tissue obtained by cultivating explants on solid medium with the appropriate mixture of plant hormones to promote and maintain the undifferentiated state. Transfer of viable *calli* to liquid medium and agitation on rotary shakers or in fermenters results in cultures of individualized cells or small aggregates of 10 to 20 cells. Alternatively, wild-type cell suspensions can be transformed with recombinant plasmids either by co-cultivation with *Agrobacterium tumefaciens*, electroporation of protoplasts, viral vectors or by particle bombardment. Several systems can be used for cultivation of plant cells such as hairy roots, immobilized cells and free cell suspensions, being the last one more suitable for large scale production in the biotechnology industry. It is possible to apply the common fermenter system to plant cell cultures with little adjustments and use standard modes such as batch, fed-batch, perfusion and continuous fermentation (Fischer *et al.*, 1999; Hellwig *et al.*, 2004).

Recombinant proteins expressed in this system are, thus, found in the culture supernatant or retained within the cells. Protein localization will depend on two factors such as the presence of targeting leader peptides in the recombinant protein and the permeability of the cell wall for macromolecules. Generally, the cytosol is not suitable for protein storage due to the presence of proteases that can degrade it. Targeting signals can be a strategy to retain the recombinant proteins within certain cell compartments. However, this creates some complications when purifying the protein, since disruption of the cell is needed prior to protein purification. This process has several drawbacks such as needing additional equipment and labor, as well as causing the release of several substances such as proteases and phenolic compounds that can reduce the final protein yield. Therefore, the preferred method is to target the protein for secretion and capture it from the culture supernatant or release it from the cell with mild enzymatic cell wall digestion (Schillberg *et al.*, 2003).

However, plant cell cultures also have some limitations like poor growth rates, somaclonal variation (chromosomal rearrangements are an important source of these variations; common in plant cell cultures generated by *calli*) and gene silencing, together with inhibition of product formation at high cell densities, formation of aggregates and wall growth as well as shear-sensitivity for some species (Fischer *et al.*, 1999; Hellwig *et al.*, 2004). Pires and colleagues (2008) have shown that a *Medicago truncatula* suspension cell culture transformed with the fungal enzyme phytase was easy to set up and that it was possible to obtain a high-level

production of the recombinant protein. They also recovered the recombinant protein easily, using different methods, and noticed that its activity was stable during the entire cell growth cycle (Pires *et al.*, 2008).

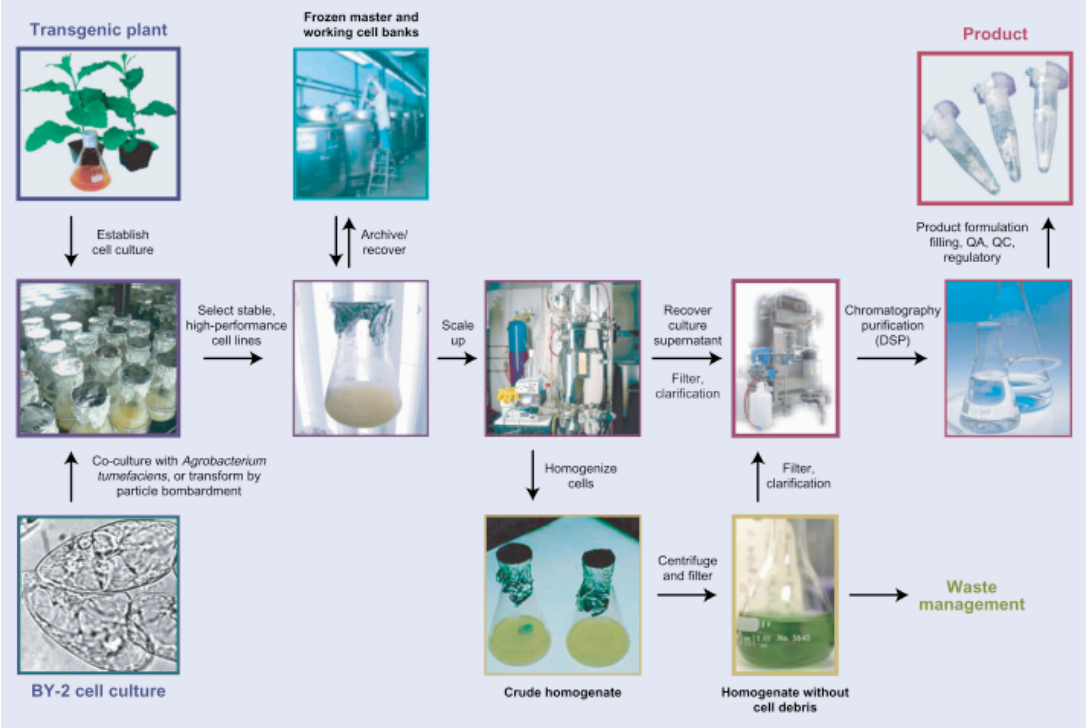

Figure 1.8.1 – How to obtain molecular farming products with transformed suspension cell cultures (Hellwig *et al.*, 2004).

The levels of expression and final product recovery in plant cell cultures are lower than the threshold where processes begin to be economically profitable. Separation of the plant cells from the fermentation supernatant is simple in comparison to bacterial fermentations and can be accomplished with several steps of filtration. In addition, some of the problems that arise from production of compounds by plant cell cultures in bioreactors can be solved optimizing the process, by improving bioreactor design and agitation conditions or by a better nutrient supply (Hellwig *et al.*, 2004). Nowadays, BY2 cells and carrot cells are being used successfully to produce recombinant proteins. James and colleagues (2000) were able to produce a human granulocyte-macrophage colony-stimulating factor (a hemopoietic growth factor) in BY2 cell cultures (James *et al.*, 2000). Shaaltiel and collaborators (2007) produced a recombinant glucocerebrosidase using carrot cell cultures (Shaaltiel *et al.*, 2007). So, suspension cell cultures seem to have a great potential as a viable system for large-scale protein production.

1.9 From Plants and Cell Cultures to the Final Product

Downstream processing is an integral part of every biomanufacturing process and it concerns the isolation and purification of the product from the raw biomass. Several aspects of downstream processing have to be customized specifically for whole-plant systems, including adding steps for the removal of fibers, oils and other by-products, along with optimizing process for the treatment of different plant species and tissues.

Nonetheless, such issues do not apply so much to plant cell cultures, where the main choice is between the extraction of proteins from the wet biomass and purification of secreted proteins directly from the culture medium. Secreted proteins are advantageous since they avoid several unit operations during the purification process and provide a starting material, the culture supernatant, which has a much lower content of contaminating proteins and other metabolites than whole cell extracts. The downside of this approach is that the recombinant protein is highly diluted, so large volumes of liquid must be processed, and that the proteins can undergo significant degradation (Hellwig *et al.*, 2004). However, when the protein is retained within a cell compartment or in the cytosol, a method suitable for cell disruption has to be applied. Mechanical cell disruption, like bead mills, arise several problems such as heat generation, disruption of cell compartments, liberation of noxious chemicals and generation of cell debris, which can be difficult to remove (Hellwig *et al.*, 2004; Pires *et al.*, 2008).

Another strategy to purify the recombinant protein, in whole plant systems or suspension cell cultures is to fuse a tag (for example polyhistidine) to the recombinant protein as it will allow the retention of the protein on purification columns specific for this tag (Hellwig *et al.*, 2004; Schillberg *et al.*, 2003).

The use of plants and cell cultures is still a growing process for the production of recombinant proteins.

1.10 Prostaglandin D2 Synthase Lipocalin-type Protein

Prostaglandin D synthase (PGDS) catalyzes the isomerization of the 9,11-endoperoxide group of PGH_2, a common precursor of various prostanoids, to produce PGD_2 with 9-hydroxi and 11-keto groups, in the presence of sulfhydryl compounds (Figure 1.10.1). This protein is a glycosylated human protein that is produced in a variety of tissues and is involved in several physiological and pathological functions. PGDS is a protein derived from the brain, being more abundant in the cerebrospinal fluid. PGD_2 can act as a potent endogenous somnogen (sleep inducer), nociceptive modulator (pain regulator), anti-coagulant, vasodilator, broncho-constrictor and as well as an allergic and inflammatory mediator released from mast cells. PGDS can also transport non-substrate small lipophilic molecules (Urade & Hayaishi, 2000).

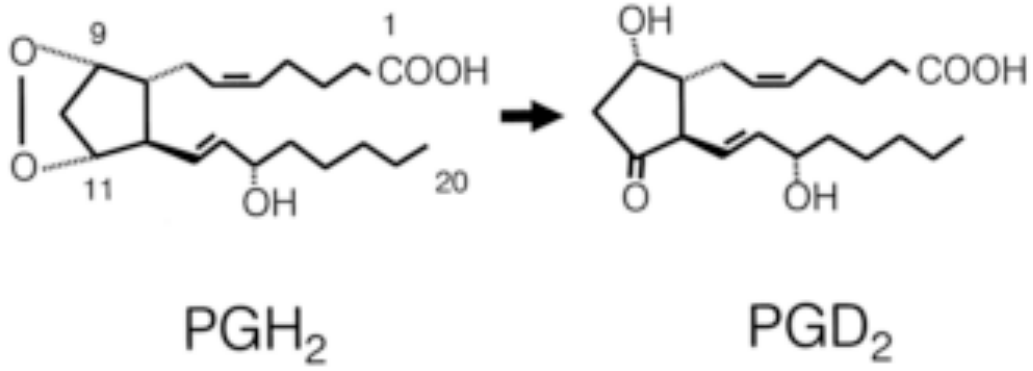

Figure 1.10.1 – Catalytic reaction by L-PGDS (Urade & Hayaishi, 2000).

Two different types of PGDS have been purified and characterized: one is the lipocalin-type (L-PGDS), which was previously known as the brain-type enzyme or glutathione (GSH)-independent enzyme, and the other is the hematopoietic PGDS, the spleen-type enzyme or GSH-requiring enzyme. These two types are different from each other in terms of catalytic properties, amino acid sequence, tertiary structure, evolutionary origin, gene structure, chromosomal localization, cellular localization, tissue distribution and also functional relevance. Besides, L-PGDS is the first member of the lipocalin family to be recognized as an enzyme and a highly glycosylated protein. Furthermore, it shares the characteristic as lipocalin, since it is secreted into various body fluids and binds small lipophilic-ligands (Urade & Eguchi, 2002).

1.10.1 L-PGDS Enzymatic properties

L-PGDS, first referred as β-trace, was discovered in 1961 by Clausen and identified as a major protein in the cerebrospinal fluid (CSF) (Clausen, 1961). When β-trace was identified as L-PGDS, the enzyme was purified from different body fluids, such as CSF and seminal plasma of humans and several mammals (Zahn *et al.*, 1993).

L-PGDS is known nowadays as an N-glycosylated monomeric protein with molecular weight of 20-31 kDa, depending on the size of the glycosyl moiety. The enzyme carbohydrate structures differ between various fluids.

This enzyme requires free sulphydryl compounds (GSH, β-mercaptoethanol, cysteine) for the reaction. Besides, it is inactivated by chemical modification of an active thiol group, Cys65, with iodocetamide or N-ethylmaleimide, and is inhibited by inorganic selenium compound and several non-substrate hydrophobic ligands such as fatty acids, retinoids, biliverdin and bilirubin. L-PGDS is a very stable enzyme and is highly resistant against heat treatment and protease digestion. However, it is easily inactivated by enzymatic reaction, probably due to oxidation of the active site during the catalytic reaction. Urade and Hayaishi (2000) suggested that L-PGDS is a multiple function protein, as it acts as a PGD_2-producing enzyme within the cell, as well as

functioning as a lipophilic ligand-binding protein after secretion into the extracellular space and various body fluids, although the endogenous ligands have not yet been identified (Urade & Hayaishi, 2000).

1.10.2 L-PGDS Tertiary Structure

Three enzyme cysteine residues, Cys^{65}, Cys^{89} and Cys^{186} are conserved among all species. The last two cysteines form a disulfide bridge, which is highly conserved among most lipocalins. On the other hand, Cys^{65} is unique to L-PGDS as it has never has been found in other lipocalins (Figure 1.10.2.1). The replacement of this cysteine with serine or alanine by site-directed mutagenesis led to complete loss of the catalytic activity of the recombinant rat, human, mouse and frog enzymes, indicating that this cysteine residue is the key residue for the reaction catalyzed by L-PGDS.

Figure 1.10.2.1 – Crystallographic structure of L-PGDS shown as a ribbon model (Urade & Hayaishi, 2000).

This enzyme is mainly composed by β-barrels with two sets of four-stranded anti-parallel β-sheets and a three-turn α-helix. Cys^{65} is located in a hydrophilic pocket with enough size to bind retinoids, biliverdin, bilirubin and thyroid hormones. This enzyme possesses a β-barrel structure with a hydrophobic pocket and, in fact, the active thiol of Cys^{65}, the active site for the catalytic reaction, faced the inside of the pocket (Urade & Hayaishi, 2000).

1.10.3 L-PGDS Glycosylation

L-PGDS is a glycoprotein, which presents two N-glycosylation sites, Asn^{29} and Asn^{56}. The structure variations in these glycosidic chains generate different isoforms modified by post-

translational processes (Lescuyer *et al.*, 2005). Hoffman and colleagues (1994) determined that all glycans were of the complex type and most (90%) of them were biantennary with no (40%), one (40%) or two (20%) N-acetylneuraminic acid residues. The 10% left were triantennary chains or biantennary chains with intact or truncated lactosamine repeats (Hoffman *et al.*, 1994).

Lescuyer and colleagues suggested that the ratio of basic and acidic forms of the enzyme is higher in Alzheimer and Parkinson patients. Acidic forms are more glycolysed, suspecting that the disease's degenerative process is associated with a deglycosylation of the protein (Lescuyer *et al.*, 2005).

1.10.4 L-PGDS Tissue and Subcelular Localization

Urade and Hayaishi determined L-PGDS tissue distribution and cellular localization in humans in 2000. They concluded that this enzyme is localized in the central nervous system and related organs, like the cochlea (inner ear constituent) and ocular tissues, in the male genital organs and heart. The enzyme produced in these organs is secreted to several body fluids, such as CSF, eye fluids, seminal plasma and plasma (Urade & Hayaishi, 2000). L-PGDS is localized in different cell types of several human organs, such as oligodendrocytes and neurons in the brain and spinal cord, marginal and basal cells of Resser's membrane in the cochlea, pigmented epithelial cells in the retina, as well as myocardial cells and endocardial cells in the heart (Lescuyer, *et al.*, 2005; Ragolia *et al.*, 2007).

Lescuyer and collaborators showed that alteration of the PGDS post-translational modification pattern in cerebrospinal fluid may be involved in some neurological disorders associated with neurodegeneration. They also hypothesized that PGDS deglycosylation may be associated with cell death, inflammation, altered blood-brain barrier, all of which known to occur in most central nervous system conditions.

1.11 Objectives

Since Molecular Farming is an important field to develop, several researchers are committed to improving recombinant protein production. Although this field is substantially developed, there are several aspects related with protein intracellular trafficking that are not well established yet. Consequently, studying and understanding the secretory pathway in plants is important to identify basic cellular processes, as well as to improve the use of plants for recombinant protein production. Our laboratory transformed *Medicago truncatula* plants with prostaglandin D_2 synthase cDNA in order to test this plant system for the production of human recombinant proteins. In this project, we aim to characterize the deposition patterns of this recombinant protein in different organs of the plant, as functional specialization of plant cells can affect the subcellular destiny of the protein (Abranches *et al.*, 2008). To achieve this we analyzed the protein expression in three different plant organs (leaf, root and seed) by Western

Blot analysis and we studied the recombinant protein subcellular localization by immunodetection using fluorescence and electron microscopy. We also aimed to understand which system (whole plants *vs.* cell suspension cultures) is more suitable for the production of L-PGDS recombinant proteins.

2 Materials and Methods

2.1 Plant Material

Medicago truncatula cv Jemalong embryogenic line M9-10a (Neves *et al.*, 1999) plants had been previously transformed in the laboratory using *Agrobacterium* mediated transformation with the pTRA-BT-AH plasmid. Transgenic seeds were available to be germinated and selected with the antibiotic kanamycin.

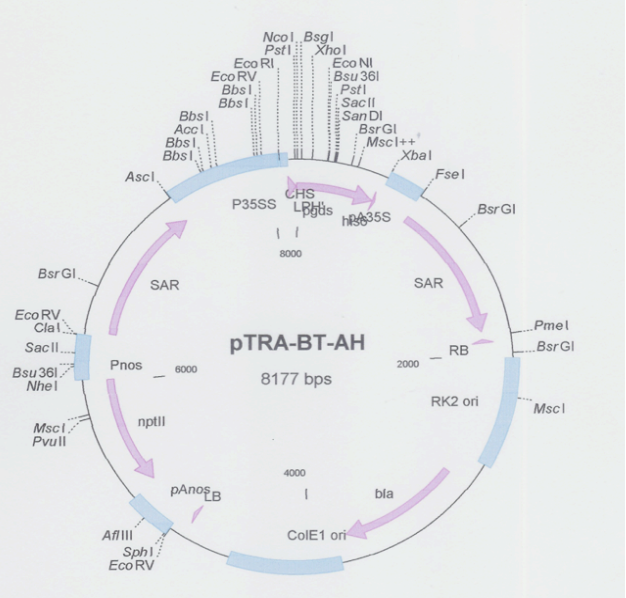

Figure 2.1.1 – pTRA-BT-AH (L-PGDS) plasmid.

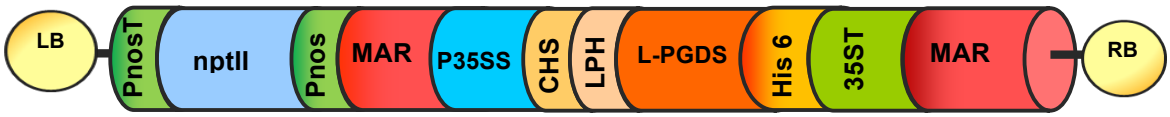

Figure 2.1.2 – L-PGDS T-DNA.

The T-DNA inserted in the plasmid, delimited by a right and a left border (RB and LB), contains the cDNA, which codifies L-PGDS protein. This cDNA is under the control of 35SS constitutive promoter (P35SS). Upstream of the cDNA is present a region that is non-translated and stabilizes messenger RNA (CHS), and also a murine leader peptide (LPH), optimized for plants, that directs the protein to the plant secretory pathway. Downstream of the L-PGDS gene is a fusion tag linked to the C-terminal with six residues of histidine (His 6) for protein

purification and a terminator sequence (35ST). There are also present MAR regions (Matrix Attachment Regions) that prevent gene silencing phenomena and stabilize the transgene expression. The cDNA also contains the kanamycin resistance cassette with the gene nptII, the promoter Pnos and the gene terminator PnosT.

2.1.1 Seed Scarification, Sterilization and Germination

The seeds need to be scarified to break the tegument. Sulfuric acid works well to achieve this step. This process is crucial to obtain a good yield of germination and it is also required to have a synchronized germination. Seeds were extracted from the pods and then placed in a 15 mL tube. In the hotte, 5 mL of sulfuric acid were added to the tube, and the tube was manually inverted for 10 minutes. Then, the sulfuric acid was removed from the tube and 10 mL of sterile distilled water were added and the tube was stirred for 5 minutes, afterwards the water was discarded and this step was repeated three to five times. In a laminar flow chamber (Faster Bio48), Petri dishes were prepared with MS Medium (see chapter 6.1) and 0.2 M kanamycin (Duchefa) for selection of transgenic seeds. Ten to fifteen seeds were placed on the medium and incubated at 23ºC for three to five days, in a 16h light period chamber.

2.1.2 Plant Transplanting and growth

After germination, plants were transferred from the Petri dish to a pot with sterile soil with vermiculite, so that plant growth could be improved by airing the soil. Plants were watered and a plastic bottle was placed in the pot covering the plant, for protection. Plants were placed in a 24ºC chamber with 16h light period. Plants were watered every other day.

2.1.3 Establishment of suspension cell cultures

Leaves from a transformed plant were removed and, for *calli* induction, wounded with a scalpel. Then, the wounded leaves were placed in Petri dishes with MS Agar culture medium (see chapter 6.1) with hormones (1mg/L of 2,4-D essential for plant body development, 1 mg/L of kinetin for cell division promotion and 10 mg/L of DTT to reduce disulfide bonds in the medium) and kept in a darkroom for a month. *Calli* were obtained at the end of this period. Then, *calli* were diced in small pieces and placed in an erlenmeyer with MS liquid culture medium with hormones. Cell cultures were left to grow in a rotative dark chamber (125 rpm) at 23ºC.

2.2 Protein Analysis

2.2.1 Protein Extraction from *Medicago truncatula* Plant Tissues

Seed, Root and Leaf:

Tissues were collected and weighted in an analytical balance (Sartorius Basic, Germany). Liquid nitrogen was added to a mortar, as well as the plant tissue. Samples were ground and the extraction buffer was added in 1:1 ratio (g of plant tissue: mL of extraction buffer). After thawing, the extraction buffer was mixed with plant tissue sample and transferred into several eppendorfs. Total protein extract was centrifuged (Biofuge 28RS, Heraeus Sepatech, Germany) at 22000 g for 20 minutes at 4ºC, the pellet was discarded and the supernatant was transferred to a new eppendorf. The supernatant was again centrifuged in the same conditions, the pellet was discarded and the supernatant obtained transferred to a new eppendorf and stored at -20ºC.

Cell culture:

The vacuum filtration apparatus was assembled and filter paper (90 mm, qualitative medium speed; Labbox, Spain) weighted. The cell culture was filtered and the filter paper with filtered cells weighted again. The cell culture medium was stored at -20ºC. Then, filtered cells were removed from the filter paper with a spatula and transferred to a mortar with liquid nitrogen. Protein extraction procedures were the same as for root, leaf and seed.

2.2.2 Sample Preparation for SDS – PAGE

The samples were prepared in 1:4 ratio (μL of Sample Buffer 4x: μL of protein extraction sample; see chapter 6.1), span down at 16200 g for 10 seconds (Centrifuge 5415 D, Eppendorf, Germany) and then boiled at 100ºC for 10 minutes in a dry bath (AccuBlock Digital Dry Bath, Labnet International, Inc.). The final mixtures were then loaded in the SDS-PAGE gel, varying the load volume according to the tissue (20 μL for leaf, 15 μL for root and 25 μL for cells and cell medium).

Sample concentration:

Cell medium and root samples were concentrated with four volumes of absolute ethanol at -20ºC overnight. The mixture was then centrifuged (Biofuge 28RS, Heraeus Sepatech, Germany) for 10 minutes at 11200 g at 4ºC. After being dried with vacuum, the resulting pellet was resuspended with sample buffer 1x and boiled for 10 minutes at 100ºC in a dry bath. The samples were then centrifuged and loaded in a SDS-PAGE gel.

2.2.3 SDS – PAGE Gel Preparation

SDS–PAGE gel was prepared in a BioRad Mini-Protean 3 Cell Electrophoresis System (Bio-Rad, USA). The resolving gel solution (12,5% acrylamide) was prepared as described in the table below, mixed carefully and then applied between gel cassette glasses. Then, the total

glass volume was filled with isopropanol as it prevents oxygen from getting in and inhibiting polymerization. The gel was left to polymerize for 1 hour and then isopropanol was removed with filter paper. The stacking gel solution was also prepared, as described below (table 1.3.1), added to the gel cassette, after which combs were placed between the glasses. The stacking gel allows sample alignment, which leads to the separation of sample proteins according to their electrophoretic mobility. After polymerization, the combs were removed.

Table 2.2.3.1 – Preparation of Resolving and Stacking Gels.

Solution	**Resolving Gel (mL) (12,5% acrylamide)**	**Stacking Gel (mL)**
Total Volume	8.88	6.16
Water	2.82	3.98
Stacking gel buffer stock (see chapter 6.1)		1.25
Resolving gel buffer stock (see chapter 6.1)	1.25	
10% SDS (Duchefa Biochemie, Netherlands)	0.1	0.05
Acrylamide:Bisacrylamide (30:0,8) (Bio-Rad, USA)	4.2	0.625
TEMED (Sigma, USA)	0.005	0.005
1,5% Ammonium Persulphate (Merck, USA)	0.5	0.25

The electrophoresis apparatus was assembled and running buffer was added until the electrodes were completely covered. Then, samples were loaded with a 50 μL syringe (Hamilton, Switzerland). 3 μL of Prestained SDS-PAGE Standards Low Range (Bio-Rad, USA) were also loaded in a separate lane. A 180 V and 30 mA (constant) electric field was applied to the apparatus for approximately 90 minutes.

One gel was colored with Coomassie G-250 solution and the other one was destined to protein transfer. The first one was left to be colored with Coomassie G-250 overnight and in the next day the de-staining solution was applied to view the protein bands.

2.2.4 Protein Transfer from SDS-PAGE gel to Nitrocellulose Membrane

The protein transfer from SDS-PAGE gel to membrane was done using the semi-dry transfer method. The filter papers (BIO-RAD), nitrocellulose membrane (Hybond-C, 0.45

micron, Amersham) and gel were immersed in protein transfer buffer before protein transfer. The sandwich was assembled on a Trans-Blot SD (Semi-dry Transfer Cell, Bio-Rad) as shown in the scheme below. 200 mA with 16 V (constant) electric field was applied to the apparatus for 25 minutes.

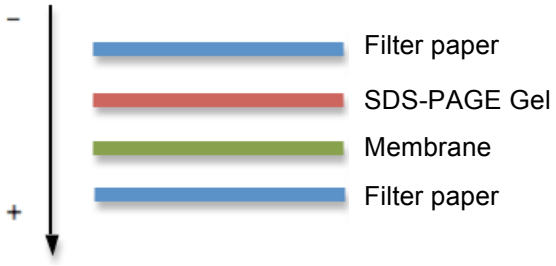

Figure 2.2.4.1 – Semi-dry transfer assembling scheme.

Figure 2.2.4.2 – Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell.

2.2.5 Protein Immunodetection

After protein transfer from the acrylamide gel to a nitrocellulose membrane, the membrane was immersed in a blocking solution (3% BSA (Sigma, USA) and 5% skimmed milk powder (Nestlé, Switzerland) prepared in PBS-T; see chapter 6.1) for 1 hour at room temperature with gentle shaking (Duomax 1030, Heidolph, Germany). After this procedure, the membrane was washed three times, 5 minutes each, with PBS-T. The primary antibody (α L-PGDS, Abcam, Ab61866) was diluted at 1:200 with PBS-T, to a final concentration of 2μg/mL. The membrane was immersed in primary antibody solution, incubated for 1 hour at room temperature and then incubated overnight at 4ºC with gentle shaking. On the next day, the membrane was washed three times, 5 minutes each, with PBS-T. The secondary antibody (α rabbit, Sigma, A3687 coupled to alkaline phosphatase) was diluted at 1:4000 with PBS-T. The membrane was immersed with the secondary antibody solution and incubated for 2 hours with gentle shaking at room temperature. In the next procedure, the membrane was washed three times, 5 minutes

each, with PBS-T, followed by NBT/BCIP detection solution (see chapter 6.1). The membrane was placed on a glass plate and the detection solution was spread on it. The membrane was covered with a black box so light did not affect the reaction. The precipitate would become visible within a few minutes.

2.3 Plant Tissue Preparation and Protein Immunodetection

2.3.1 Microscopy slides Treatment with Poly-L-Lysine

Glass slides with eight wells (Thermo Scientific, USA) were washed with a 2% Decon solution (Decon-9, Decon Laboratories Limited, United Kingdom) and bi-distilled water in a 1:50 ratio. Slides and washing solution were placed in a coplin jar stirring for 2 h. Then, the slides were washed with bi-distilled water and air-dried. Poly-L-Lysine (Sigma-Aldrich, USA) solution was prepared in 1:10 ratio in bi-distilled water, so that 800 μL of solution could be placed on each slide, to ensure tissue and cell adherence. The solution was applied on the slides and left for 5 minutes. The poly-L-Lysine excess was then removed and the slides were left to dry overnight at room temperature.

2.3.2 *Medicago truncatula* Plant Tissues Fixation

Root and Seed Fixation:

Root tips and seeds were fixed in 15 mL tubes with 4% paraformaldehyde (Merck, USA) fixation solution freshly prepared, stirring for 1h30 to 2h. The tissues were then washed 2 times for 10 minutes with TBS followed by bi-distilled water, so that TBS buffer salts can be removed.

Leaf Fixation:

Before the fixation process, leaves have to be discolored, since chlorophyll auto-fluorescence interferes with the microscopy results. For this purpose, two ethanol (Panreac, Spain) solutions were prepared in bi-distilled water, one with 70% ethanol and the other with 50% ethanol. The leaves were cut longitudinally in halves with a scalpel and added to 10 mL tubes with 70% ethanol solution. The plant tissue was incubated in a water bath (Grant GD100, United Kingdom) at 70ºC for 10 minutes. Then, the 70% ethanol solution was discarded and the 50% ethanol solution was added to tubes, stirring on hand for 1 minute. 50% ethanol solution was then discarded and leaves were placed in distilled water to prevent dehydration (Erbs *et al.*, 2008). The leaf fixation procedure was the same as described for root fixation.

2.3.3 *Medicago truncatula* Plant Tissue Section

For this procedure, a vibratome (1000 plus Sectioning System, Vibratome, USA) was used and 30μm sections were cut.

Figure 2.3.3.1 – Vibratome 1000.

Root Sections:

Root tips were removed from tubes where fixation was performed and placed in a Petri dish. Then, tips were glued to a solid cube with super glue (Loctite, Henkel, Germany) and the set was placed on the tightened solid support. Then, water was added to the tray, covering the blade and the solid support. Speed and amplitude were set to 6,5 and 7,5 respectively. From controlling the direction control switch, the pause switch and the section thickness switch, root sections were obtained. The root sections were cut with 30 μm thickness.

Leaf Sections:

To obtain the leaf section, leaf halves (from fixation) had to be immobilized in 2,5% agar (Duchefa Biochemie, The Netherlands) cubes, by inserting the agar solution in a pharmaceutical blister packaging and then placing the leaf halves. The procedures were the same as for obtaining root sections, except for the speed parameter that was changed to 2.

Seed Sections:

Seed coats were removed with tweezers and the cotyledons were separated from each other, dividing the embryo in halves. The following procedures were the same as for root sections.

2.3.4 Immunodetection of Recombinant L-PGDS in Suspension Cultured Cells by Fluorescence Microscopy

1 mL of cell culture was transferred from an erlenmeyer to a test tube. Samples were centrifuged at 0,1 g for 10-15 minutes, the excess medium was removed, and at least 2x the cell volume of 4% paraformaldehyde were added. Then, the cells were left in the fixative, stirring for 1h. Samples were centrifuged again with the same parameters as before and the fixative was removed. Cells were rinsed gently by resuspension in PBS and then pelleted for removal of PBS. A couple of drops were placed onto poly-L-lysine treated slides and cells were allowed to settle for 10-15 minutes. The unbound cells were wicked away with a piece of paper towel. 2% Cellulase R10 in 1x TBS for 2 hours was applied to the sample. Slides were stirred in a coplin

jar with PBS solution for 10 minutes. After that, PBS excess was wicked away and blocking was performed by treating cells with 3% (w/v) BSA for 15 minutes. Then, slides were incubated with anti – L-PGDS antibody (2μg/mL) diluted in 3% (w/v) BSA in PBS 1x for at least 45 minutes at room temperature in a humid chamber. Then, slides were rinsed in fresh PBS for 15 minutes. PBS excess was wicked away and cells were treated with an appropriate secondary antibody anti-rabbit (40μg/mL) diluted in 3% (w/v) BSA in PBS 1x for at least 45 minutes at room temperature. Slides were rinsed in fresh PBS for 15 minutes. PBS excess was removed and DAPI was added and left for 5 minutes in a humid chamber. DAPI excess was removed and antifading mounting medium VectaShield (Vector Laboratories, Inc., USA) was added. A coverslip was applied, and then slides were sealed and kept at 4ºC (Paddock, 1999).

2.3.5 Immunodetection of Recombinant L-PGDS in Root, Leaf and Seed by Fluorescence Microscopy

2% Cellulase R10 (Duchefa Biochemie, The Netherlands) in 1x TBS were added to the slides and cell wall permeabilization was performed for 2 hours in a humid chamber since it allows the antibody access inside the cell. Slides were washed in a coplin jar with PBS for 10 minutes, and the excess was removed from the slide wells. Then, primary antibody anti – L-PGDS solution was prepared in 3% (w/v) BSA to a final ratio of 1:50 and, then, applied to the slides and blocking was performed for 45 minutes in a humid chamber. The slides were washed in fresh PBS three times for 5 minutes each. Then, secondary antibody was also prepared (40μg/mL) in 3% (w/v) BSA and applied to slide wells for 45 minutes, in a humid chamber. Slides were washed again with PBS three times, 5 minutes each, and then DAPI solution was applied to slide wells in a humid chamber for 5 minutes and excess removed with vacuum. Finally, antifading mounting medium Vectashield was applied to the slides. For visualization of the tissues and the protein deposition, three microscopes were used: Leica DMRB Fluorescence Microscope, Leica DM600B Fluorescence Microscope and Leica SP5 Confocal Microscope, Germany. For the first microscope, an UV lamp was used with N2.1 and A/DAPI filters and the camera used was Leica DFC 340 FX. For the second microscope, an UV lamp was also used with Texas Red and DAPI filter and the camera used was Andor Technology, model DU-885K-CSO-VP. For the confocal microscope, the lasers used were argon, DPSS 561 and HeNe 633 and images were acquired with the confocal system Leica TSC SP5. Images obtained were treated with FIJI® program for Z and 3D projections and Adobe Photoshop CS® program for overlaying images.

Figure 2.3.5.1 – Leica DMRB Fluorescence Microscope (A), Leica DM600B Fluorescence Microscope (B) and Leica SP5 Confocal Microscope (C).

2.4 Immunodetection of Recombinant L-PGDS in Root, Leaf and Seed by Electron Microscopy

2.4.1 *Medicago truncatula* Plant Tissue Fixation for Immunolocalization

One 16% paraformaldehyde ampoule (Electron Microscopy Science, USA) was diluted in PBS 1x to reach the final concentration of 4% paraformaldehyde in PBS. Then, it was aliquoted in 1,5 mL eppendorf tubes and frozen at -20ºC. The tissue was placed in a Petri dish with 3 mL of fresh fixative and cut it small pieces. Then, with a brush, it was placed in an eppendorf with fresh fixative. When the samples did not settle at the bottom of the eppendorf, they were placed at a vacuum chamber for the sample to be submersed and the fixative can act. Eppendorf tubes were left overnight at 4ºC. The next day, three washes with PBS 1x were made; 30 minutes each at 4ºC. Samples were kept at 4ºC until they were processed.

2.4.2 *Medicago truncatula* Plant Tissue Preparation

This procedure was done using a freeze substitution and progressive lowering of temperature Leica EM AFS2 embedding system. In the first day, the AFS apparatus was filled with liquid nitrogen. In the chamber of EM AFS2 three sample containers were placed with plastic capsules where the samples were added together with 30% methanol. The AFS system was programmed for sample dehydration on the first day as described in the following table.

Table 2.4.2.1 – Leica EM AFS2 First Day Setting.

Temperature (ºC)	Time	Solution
0	2 h	30% methanol
-15	2 h	50% methanol
-30	2 h	70% methanol
-30	Overnight	100% methanol

The next day, EM AFS2 was programmed for substitution of the methanol by the embedding resin Lowicryl, as shown in the table 2.4.2.1.

Table 2.4.2.2 – Leica EM AFS2 Second Day Setting.

Temperature (ºC)	Time	Solution
-30	30 min	75% methanol/25% Lowicryl
-30	1 h	50% methanol/50% Lowicryl
-30	1 h	25% methanol/75% Lowicryl
-30	1 h	100% Lowicryl
-30	Overnight	100% Lowicryl

The third and the fourth day, EM AFS2 was programmed for the embedding sample in the Lowicryl resin, as shown in the next table. In every step, old resin was replaced with a freshly made one.

Table 2.4.2.3 – Leica EM AFS2 Third and Fourth Day Setting.

Temperature (ºC)	Time	Solution
-30	4 h	100% Lowicryl
-30	4 h	100% Lowicryl
-30	Overnight	100% Lowicryl
-30	4 h	100% Lowicryl
-30	4 h	100% Lowicryl
-30	Overnight	100% Lowicryl

The fifth day, EM AFS2 was programmed for resin polymerization and, therefore new Lowicryl resin was freshly prepared. In new sample containers, gelatin capsules were added and 6 drops of freshly prepared resin were added to each capsule. With a specimen carrier-detaching tool, plastic capsules were inserted into gelatin capsules. The sample containers were filled with 15 mL of ethanol in order to prevent overheating of the sample. EM AFS2 was programmed as described in the following table.

Table 2.4.2.4 – Leica EM AFS2 Fifth Day Setting.

Temperature (ºC)	Time
-30	64 h
0	3 h
11	1 h
22	48 h

A LED-based UV lamp lid was placed on top of the capsules and turned on until the program ended. Then, capsules were released and placed in the sunlight for 24 hours for finishing the polymerization process.

2.4.3 *Medicago truncatula* Plant Tissue Sectioning

Plant tissue sections must be analyzed first by optic microscopy in order to decide whether the tissue is in good conditions to proceed to electron microscopy analysis. These first sections are made using a glassknife made from cutting a glass bar (previously washed with soap, ethanol and distilled water, always with gloves, so the hand grease is not transferred to the bar) in a glass knifemaker (Leica EM KMR2, Germany). Then, in an ultramicrotome (Leica EM UC6, Germany), the tissue embedded in Lowicryl resin was sectioned in slices of 100 nm with the glassknife. Finally, the tissue section was dyed with toluidine blue (colors cell walls, cytoplasm and nucleus) and observed under a Nikon Eclipse E1000 light microscope. If the tissue section seems well fixated and embedded, the sectioning will be carried out for observation under the electron microscope. In this case, a Diatome 45º diamond knife is used to cut 100 nm sections in the ultramicrotome. Tissue sections are placed in grids and left to dry.

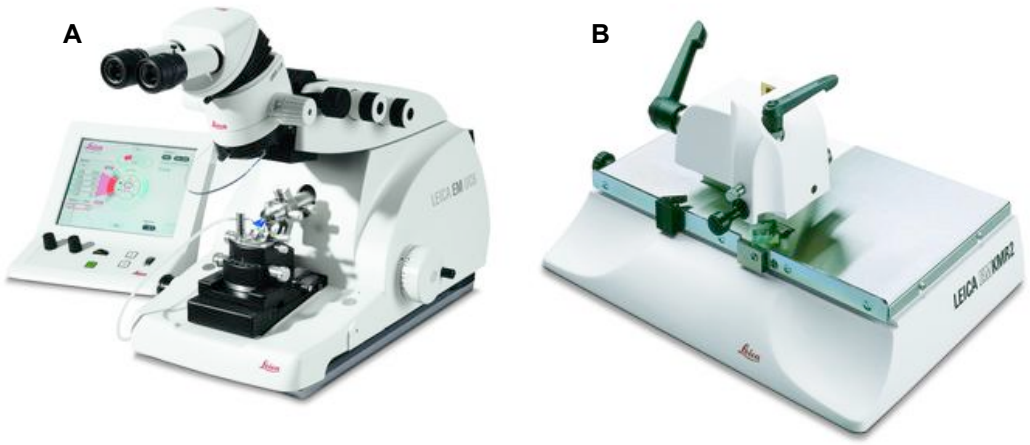

Figure 2.4.3.1 – Ultramicrotome Leica EM UC6 (A) and Glass Knife maker Leica EM KMR2 (B).

2.4.4 Immunodetection of Recombinant L-PGDS in *Medicago truncatula* Plant Tissue

A piece of parafilm was cut with 10 cm length and 5 cm wide and wells were made using the back of a pencil (as shown in figure 4.4.1). Parafilm was placed in a squared Petri dish as well as a wet piece of paper to create a wet chamber. In each line of wells of the parafilm some drops of each reagent were placed. Thus, the grids with the sections made earlier were transferred from a drop to another. First the grids were placed in miliQ water for 3 minutes, followed by 3 minutes in autoclaved PBS 1x. For blocking unspecific reactions, grids were transferred to drops of 5% BSA in PBS 1x for 15 minutes. Then, grids were dried using filter paper before being transferred to anti – L-PGDS primary antibody (1/10) drops diluted in 1% BSA for 3 hours at 25ºC. Following the 3 hours of primary antibody incubation, grids were placed in drops of PBS 1x for washing, 6 washes of 5 minutes each. Grids were then dried with filter paper before being placed in anti–rabbit 10 nm gold particle secondary antibody (BB International, UK), diluted at 1/25 with 1% BSA. Secondary antibody incubation was performed for 1 hour at 25ºC. Grids were washed again with PBS 1x, 6 washes of 5 minutes each, and then placed in drops of PBS 1x and 2% glutaraldehyde for 10 minutes. Finally, grids were washed for 5 minutes in PBS 1x and then washed two times for 5 minutes in miliQ water. Grids were left to dry.

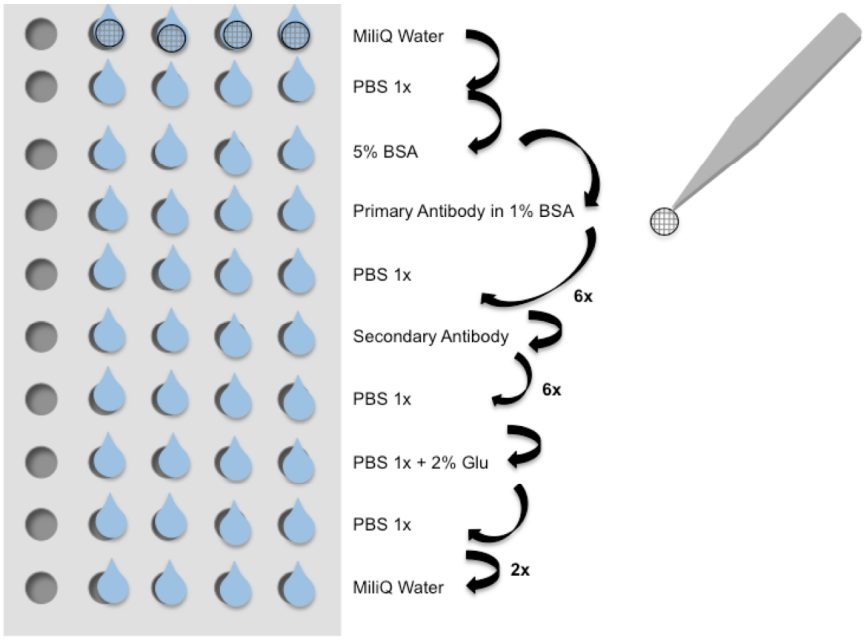

Figure 2.4.4.1 – Immunolocalization scheme.

Then, the sections in the grids have to be counterstained with 0,5% uranyl acetate (Electron Microscopy Sciences, USA) and lead citrate solution (Electron Microscopy Sciences, USA; see chapter 6.1) to increase the sample contrast when seen on electron microscope. The scheme

used to do this final procedure was the same for immunodetection. First, grids were placed in drops of uranyl acetate for 10 minutes, followed by twelve washes with miliQ water. Then, grids were transferred to lead citrate solution for 10 minutes and finally washed twelve times with miliQ water. Grids were stored at room temperature. Samples were visualized under an electron microscope (Philips CM10, The Netherlands) and photos taken with a CCD camera (Gattan, UK).

2.5 Electron Microscopy analysis of *Medicago truncatula* Tissue Structure and Recombinant Protein Deposition Pattern

The work that will be described here was developed at the Cellular Biology Lab at Instituto de Conservación y Mejora de la Agrodeversidad Valenciana, Universidad Politécnica de Valéncia, under the supervision of Dr José Maria Simarro, from March to June 2012.

2.5.1 Fixation of Plant Material

3 mL of Karnosvky were transferred to a Petri dish that had been previously placed in ice. The sample was placed in the fixative and cut in small pieces. With a brush, samples were transferred to an eppendorf with fresh fixative. When the samples did not settle in the bottom of the eppendorf, they were placed in a vacuum chamber for some minutes until they are totally submersed. Then, samples were left at room temperature for 3 hours, after which the fixative was replaced for a freshly prepared one and the samples were left at 4ºC overnight. The following day, samples were washed with 0,025M cacodylate and 1% sucrose three times for 30 minutes each. Then, osmium solution was added and eppendorfs were placed at 4ºC for 5 hours. This step improves fixation, since it avoids lipid extraction. The membranes become more permeable and, therefore, the osmotic changes do not affect the cell for the following process. This osmium solution also acts as a contrast agent in proteins, nucleic acids and in membrane polar lipids, allowing the visualization of the biologic membranes.

2.5.2 Plant Tissue Preparation for EM Analysis

The samples were processed using an Electron Microscopy Tissue Processing apparatus Leica EM TP. On a sheet of paper, the several samples to process were distributed in the eight compartments of each basket as shown in figure 6.4.2.1, for easy identification of the samples after inclusion. The baskets were distributed in the wells of the flat baskets and a solution of 30% ethanol was transferred to the baskets. Samples were placed in each division of the basket. With a spike, the different baskets were put together, starting with basket no.1 and ending with basket no.7. Finally, baskets were tightened together with an aluminum lid with the help of a basket stem assembly and placed in a vial with 30% ethanol and finally transferred to the Leica apparatus. Here, the ethanol solution was replaced by epon resin.

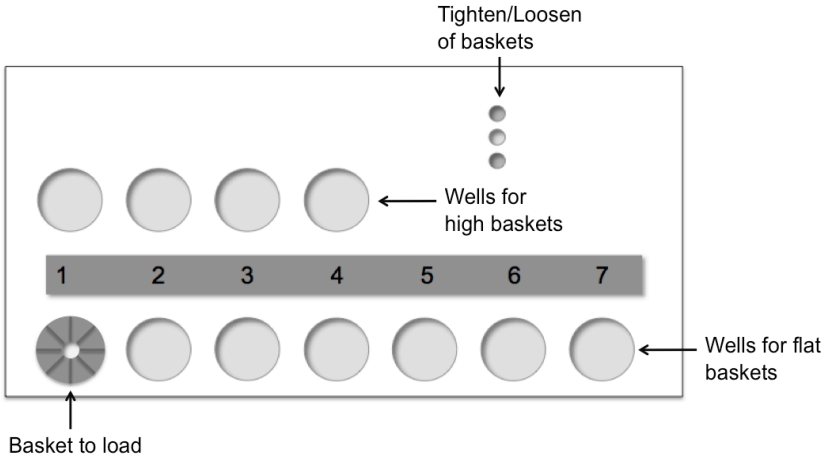

Figure 2.5.2.1 – Loading plate scheme.

The Leica apparatus was programmed as described in the following table.

Table 2.5.2.1 – Epon resin embedding program.

Solution	Temperature (ºC)	Time	Objective
30% Ethanol	4	30 min	Dehydration
50% Ethanol	4	30 min	
70% Ethanol	4	Overnight	
70% Ethanol + 1% Uranyl Acetate	4	2 h	Dehydration + TEM contrast
90% Ethanol	4	10 min	Dehydration
96% Ethanol	4	30 min	
100% Ethanol	4	2 x 1 h	
100% Ethanol 3:1 Epon	4	3 h	Infiltration
100% Ethanol 1:1 Epon	4	Overnight, stirring	
100% Ethanol 1:3 Epon	4	3 h, stirring	
100% Epon	4	Overnight, stirring	
100% Epon	4	3 h, stirring	
100% Epon	4	Overnight, stirring	
	35	2 h	Remove moisture

Fresh epon resin was prepared and added to gelatin capsules (one capsule per sample). After two hours in the incubator at 35ºC, samples were transferred from the baskets to the

gelatin capsules with fresh resin. Finally, gelatin capsules were placed in a incubator for 24 hours at 60ºC to initiate resin polymerization.

2.5.3 Plant Tissue Sectioning and Observation under EM

The sectioning of the tissue embedded with epon resin was carried out in the same way as for lowicryl resin (see chapter 4.3). Then, grids were observed under the electron microscope (Philips CM10, The Netherlands).

3 Results and Discussion

3.1 Protein Expression Level Analysis

3.1.1 Leaf and Root L-PGDS Protein Extraction Analysis

From plants fully-grown, leaf and root extractions were prepared as mentioned in materials and methods with the purpose of analyzing the expression of L-PGDS in these two tissues. After the extraction, the samples were applied in an SDS-PAGE gel, which separates proteins according to their electrophoretic mobility. The results of this procedure are shown in the following figures.

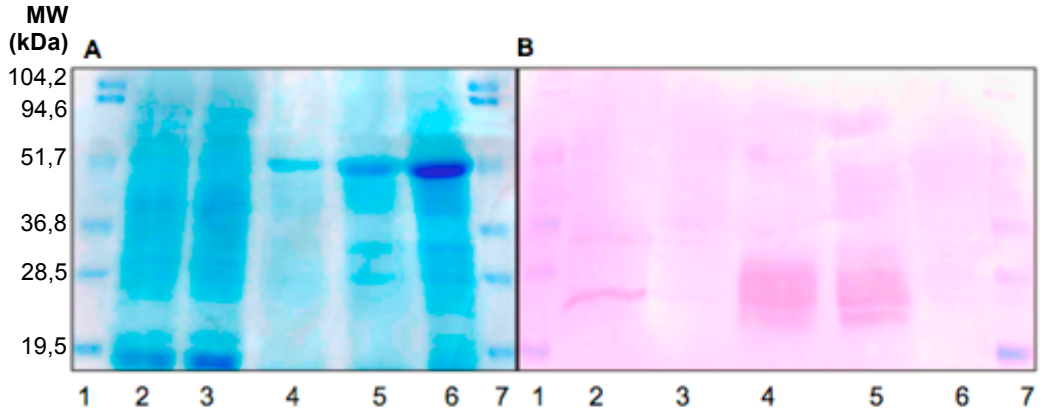

Figure 3.1.1.1 – Leaf and Root L-PGDS Protein Extraction Analysis. SDS-PAGE (A) and Western blotting (B) of leaf and root protein extractions. Lane 1 and 7 – Marker Low Range BIO-RAD, lane 2 – transgenic Root, lane 3 - WT root, lane 4 – transgenic Leaf from plants in pots, lane 5 – transgenic leaf from *in vitro* plants, lane 6 – WT leaf.

The SDS-PAGE electrophoresis gel was prepared as described in materials and methods, chapter 2.2.

The sample volume loaded in the gel was different for the two samples: root and leaf. For leaf samples 20 μL were loaded and for root samples 10 μL.

In the electrophoresis gel, the lanes 4, 5 and 6 corresponding to the leaves, present a protein distribution characteristic of leaves, with a strong band corresponding to rubisco, in contrast to the root lanes (2 and 3), where this protein is not present. In the root sample, the bands in different lanes are of the same intensity showing that the amount of protein in each sample was similar. In contrast, the leaf sample lanes are of different intensity due to different protein concentration in each sample.

On the nitrocellulose membrane, lane 2 presents a single band with the molecular weight expected for L-PGDS protein (≈ 26 kDa), showing that in the root tissue, probably, there is a single glycoform of this protein. Regarding lane 3, *Medicago truncatula* wild type root, there is no visible signal, confirming that the primary antibody is specific for the recombinant protein in study and that the L-PGDS band in lane 2 is specific to the transformed tissue, since there are

no unspecific reactions of the antibody to the protein. Leaf lanes 4 and 5 present an intense band with the molecular weight expected for the L-PGDS around 26 and 29 kDa, in contrast with the wild type lane, lane 6, which does not present any band. When comparing root and leaf bands, we can conclude that the root tissue sample is more homogeneous than leaf tissue sample since it only presents a single band.

There are several studies comparing the expression of a given protein in different plant organs. For example, in 2006, Drakakaki and colleagues studied the expression of heterologous phytase in *Oryza sativa* in leaf and seed tissues. They also observed by Western blotting that the resulting bands were different between the tissues. In the nitrocellulose membrane is it clear that leaf and seed tissue bands differed in molecular weight. Seed sample resulted on a single blurred band (65 kDa) and, in contrast, the leaf sample presented two bands corresponding to two forms of the recombinant protein with two different molecular weights (65 and 75 kDa) (Drakakaki *et al.*, 2006). In 2008, Abranches and colleagues also studied the expression of phytase in leaf and seed tissues, but using the plant model *Medicago truncatula*. Their observations showed that phytase expressed in seed showed a single blurred band with 65 kDa and when expressed in leaves the nitrocellulose membrane showed a double band with 65-70 kDa (Abranches *et al.*, 2008). These differences of expression of a recombinant protein in different plant organs shows that for each protein, the expression differs between the organ where it is expressed and, for different proteins, the pattern of expression in different organs also differs.

3.1.2 Seed L-PGDS Protein Extraction Analysis

The plants from which seeds would be removed to proceed to protein analysis died, so seed protein analysis was performed using dried stored seeds. The SDS-PAGE gel and the western blotting were performed as described in the materials and methods section 2.2. The sample volume loaded in the SDS-PAGE gel was 25 μL.

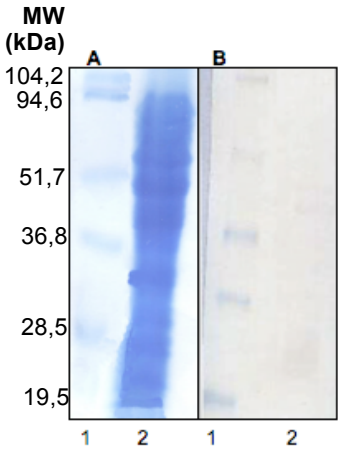

Figure 3.1.2.1 – Seed L-PGDS Protein Extraction Analysis. SDS-PAGE (A) and Western blotting (B) of seed protein extractions. Lane 1 – Marker Low Range BIO-RAD, lane 2 – transgenic Seed.

The SDS-PAGE gel bands are very intense due to a higher concentration of proteins extracted. On the nitrocellulose membrane, it is possible to notice a blurred band of low intensity with the expected molecular weight for L-PGDS (26kDa). The low intensity of the band is probably due to the fact that the seeds used for the protein analysis were a mixture of first generation transgenic seeds, including both transformed and non-transformed seeds. Future work will be carried out using seeds from a tested transformed plant in order to obtain more conclusive results.

3.1.3 L-PGDS Protein Extraction Analysis from Cell Culture Medium

1,5 mL of cell culture medium was removed from the erlenmeyer one month after the suspension culture was established. Then, using the procedures mentioned earlier, the expression of L-PGDS in this system was analyzed.

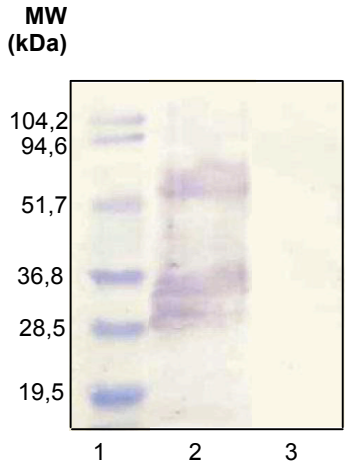

Figure 3.1.3.1 – Western Blotting of Cell Medium Protein Extraction. Lane 1 – Marker Low Range BIO-RAD, lane 2 – transgenic Cell Medium, lane 3 – wild type Cell Medium.

The western blotting was prepared as described in materials and methods section 2.2.
The sample was concentrated 20 times and the volume loaded in the SDS-PAGE gel was 25 μL.

It is possible to notice a blurred band around 28 to 36 kDa of molecular weight, which is higher than what was expected (26kDa). It is also possible to notice a higher molecular weight band of approximately 52 kDa, which is almost the double weight of the lower band, suspecting of a dimmer formation. However, L-PGDS is described in the literature as a monomer (Urade & Hayaishi, 2000) and the only PGDS described in the literature as a dimer is the hematopoietic PGDS (H-PGDS) (Urade *et al.*, 2006).

3.2 L-PGDS Subcellular Deposition Analysis by Fluorescence Microscopy

Subcellular deposition of each plant tissue was analyzed by sectioning fixed tissues using a vibratome, as described previously, and submitting them to a fluorescence immunodetection. The purpose for the process is to understand where the protein L-PGDS accumulates in the different tissues. To achieve this, for each tissue three histological sections were obtained, one from wild type plants and two from transformed plants, applying the specific primary antibody to only one of the sections of the transformed plant. This way, it will be possible to deduce if the primary antibody is specific for L-PGDS and if the signal of the transformed tissue treated with the primary antibody is in fact indicating the presence of the protein L-PGDS and not any other protein. Results for each tissue are depicted in the following figures.

3.2.1 Analysis of Protein Deposition Patterns in the Leaf Tissue

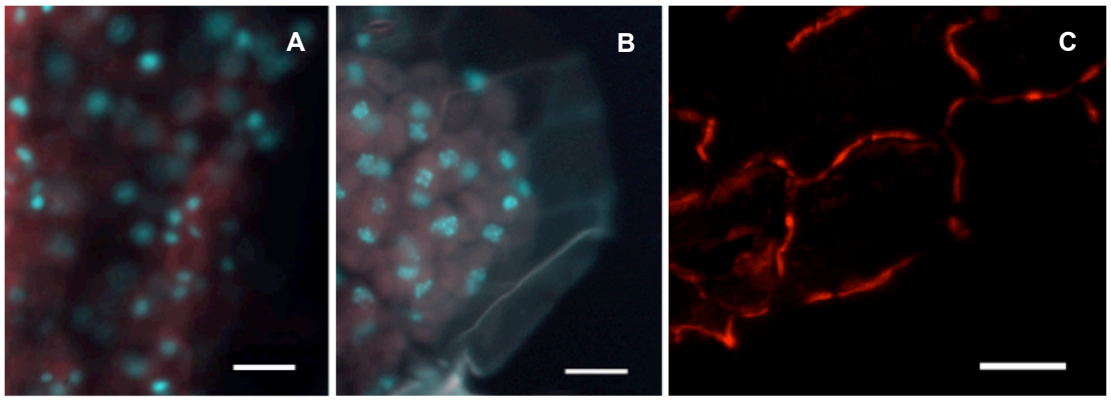

Figure 3.2.1.1 – Analysis of Protein Deposition Patterns in the Leaf Tissue. Wild type leaf with primary and secondary (A), transgenic leaf without primary antibody and with secondary antibody (B) and transgenic leaf with primary and secondary antibody (C); red – L-PGDS protein; blue – DAPI. Figures A and B were taken with a Leica DMRB Fluorescence Microscope and figure C with Leica SP5 Confocal Microscope. Scale bar = 15 μm.

The images presented in the figure above were obtained as described in materials and methods chapter 2.3. Samples analyzed by fluorescence microscopy were prepared in two groups: wild type and transformed plants treated with L-PGDS specific primary antibody and the other group did not receive primary antibody treatment to serve as a negative control.

By analysis of figure 3.2.1.1 it is possible to infer that the primary antibody is specific for the protein under study since the figure corresponding to wild type leaf (A) and the figure corresponding to transgenic leaf without primary antibody (B) present only a background, non-specific, red signal. In figure C it is possible to visualize the antibody signal in red, showing that the L-PGDS protein is being accumulated in the apoplast of the leaf cells. DAPI allows visualization of the cell nucleus.

Comparing with figure 3.1.1.1 A and B lanes 4 to 6, it is possible to deduce that, in leaf tissue, the recombinant protein only accumulates in the apoplast and, probably, in only one glycoform, since in the nitrocellulose membrane (B) it is possible to see only one intense band.

In 2001, Peeters and colleagues observed that a Fab fragment was targeted for secretion and accumulated in the apoplast in *Arabidopsis thaliana*, when a signal for retention was absent (Peeters *et al.*, 2001). In 2004, Arcalis and colleagues, studied the accumulation of phytase in wheat plants, observing that in the absence of any targeting signal, the recombinant protein accumulated in protein bodies inside the cell, in contrast with what Peeters and we observed (Arcalis *et al.*, 2004).

By analyzing these studies we can conclude that accumulation of a certain recombinant protein can vary due to plant species used in the study (every plant species has its particularity so recombinant protein accumulation can vary), the recombinant protein itself, and the plant organ where it is expressed.

3.2.2 Analysis of Protein Deposition Patterns in the Root Tissue

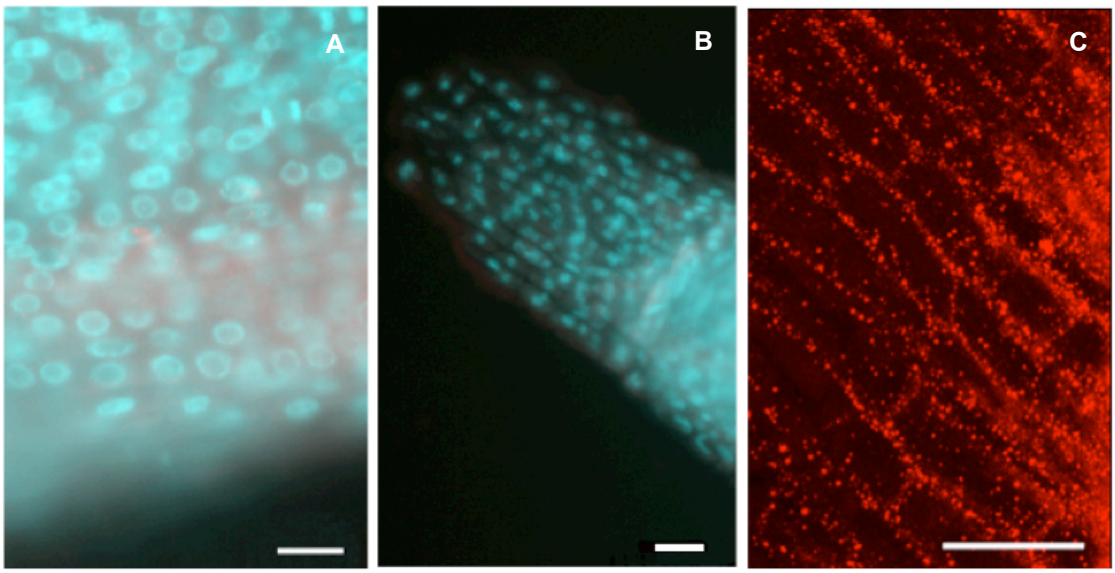

Figure 3.2.2.1 – Analysis of Protein Deposition Patterns in the Root Tissue. Wild type root with primary and secondary antibody (A), transgenic root without primary antibody and with secondary antibody (B) and transgenic root with primary and secondary antibody (C); red – L-PGDS protein and blue – DAPI. Figures A and B were taken in Leica DMRB Fluorescence Microscope and figure C with Leica SP5 Confocal Microscope. Scale Bar = 15 μm (A and C) and 10 μm (B).

The images above were obtained as described in materials and method section 2.3. The root samples were prepared also in two groups as described for the leaf samples.

As for figure 3.2.1.1 is also possible to infer that the primary antibody is specific for the protein under study in the root tissue, since figures A and B do not show any signal, only

background. By analysis of figure C it is possible to deduce that L-PGDS accumulates in the apoplast, probably in the form of vesicles, since it is possible to visualize the signal in the form of dots. In 2001, Peeters and colleagues also observed that the Fab fragment that they were studying accumulated in the apoplast of *Arabidopsis thaliana* roots (Peeters *et al.*, 2001). However, the dot pattern observed in *Medicago truncatula* root apoplast is not observed in Peeters' study.

Comparing figure 3.2.2.1 with the figure 3.1.1.1, lanes 2 and 3, it can be deduced that, as for leaf tissue, the protein accumulates in the apoplast and, probably, has only one glycoform since there is only one band in figure 3.1.1.1 B. This band is well defined suggesting that the recombinant protein produced in the root is very homogeneous, most likely with only one glycoform.

3.2.3 Analysis of Protein Deposition Patterns in the Seed Tissue

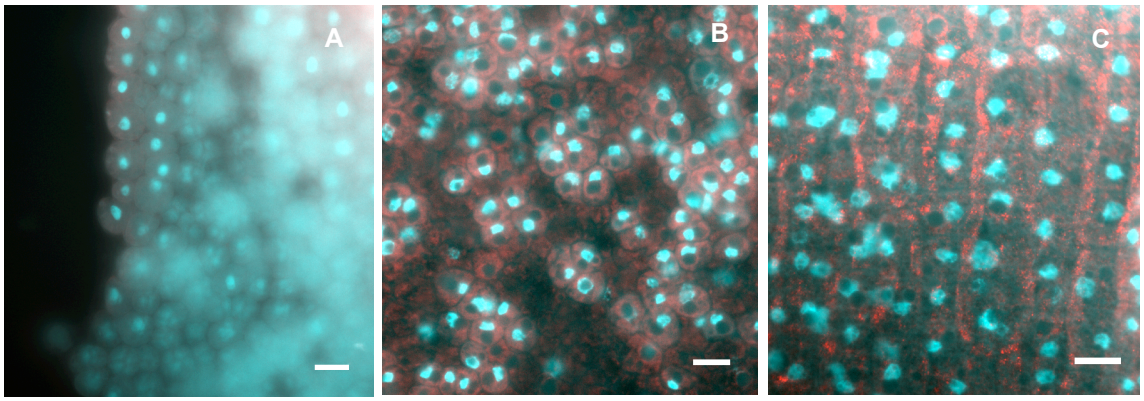

Figure 3.2.3.1 – Analysis of Protein Deposition Patterns in the Seed Tissue. Wild type seed with primary and secondary antibody (A), transgenic seed without primary antibody and with secondary antibody(B) and transgenic seed with primary and secondary antibody (C); red – L-PGDS protein and blue – DAPI. Figures were acquired using a Leica DMRB Fluorescence Microscope. Scale bar = 15 μm.

The images above were obtained as described in the material and methods chapter 2.3. The seed samples were prepared also in two groups as described for the leaf samples.

By observing images A and B of figure 3.2.3.1 it is possible to deduce that the primary antibody is specific for L-PGDS protein. The pattern observed in the transformed seed (C) is not obvious, but It seems that the protein accumulates outside the cell. Drakakaki and Abranches also studied the deposition pattern by fluorescence microscopy of phytase in rice and in *Medicago truncatula*, respectively. Drakakaki observed that phytase accumulated in the protein storage vacuoles in the rice endosperm (Drakakaki *et al.*, 2006) and Abranches noticed that the phytase signaled to the secretory pathway was accumulating in the apoplast of cotyledon cells (Abranches *et al.*, 2008). These observations suggest that, once more, the intracellular fate of a certain protein depends on the plant where it is produced.

When comparing these images with the western blotting in the section 3.1.2 (figure 3.1.2.1) there is not a clear correspondence because different accumulation sites would correspond to multiple bands. Besides a glycoform analysis, it would be useful to observe seed sections under the electron microscope in order to understand where the protein localizes exactly.

3.2.4 Analysis of Protein Deposition Patterns in the Cultured Cells

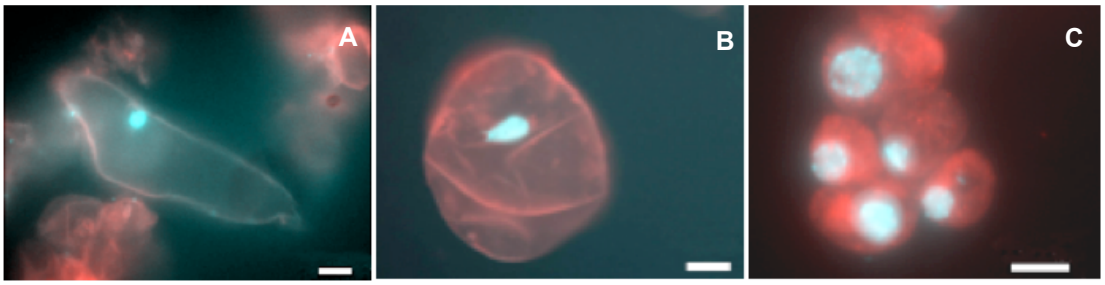

Figure 3.2.4.1 – Analysis of Protein Deposition Patterns in the Cultured Cells. Wild type cell with primary and secondary antibody (A), transgenic cell without primary antibody and with secondary antibody (B) and transgenic cell with primary and secondary antibody (C); red – recombinant protein; blue – DAPI. Figures A and B were acquired with a Leica DMRB Fluorescence Microscope and figure C with Leica SP5 Confocal Microscope. Scale bar = 15 μm.

The images above were obtained as described in chapter 2.3, material and methods.

Once more it is possible to observe that the primary antibody is specific for the L-PGDS protein. The pattern observed in the image C is totally different from any other observed, being similar with little dots. Probably the protein is being accumulated in small vesicles until it is secreted from the cell to the medium. When these images are compared with the nitrocellulose membrane in section 3.1.3 (figure 3.1.3.1) there is not a match for the protein band detected (28,5 kDa) since it seems to be several possible localizations of the protein in the cell. To better understand these patterns, it would help to perform a glycoform analysis and to observe these cells under the electron microscope, in order to understand if the protein is accumulating in vesicles or other structures.

3.3 Subcellular Deposition Analysis of L-PGDS by Electron Microscopy

Protein deposition of leaf and root tissues was analyzed by sectioning fixed embedded tissues with an ultramicrotome, as described above, and submitting them to immunodetection with 10nm gold particles. The purpose of this procedure is to understand where the protein L-PGDS is accumulating in the cell. To accomplish this, for each tissue two histological sections were obtained, one from the wild type plant and the other one from the transformed plant, followed by application of the anti - L-PGDS primary antibody and the secondary antibody with

gold particles. In this way, it will be possible to see where the protein is accumulating with great detail by observation of the gold particles on the tissue

The EM work described in this chapter was developed in the Cellular Biology Lab in the *Instituto de Conservación y Mejora de la Agrodeversidad Valenciana* (Valencia, Spain), under the INTERBIO's exchange program.

Results for each tissue are depicted in the following figures.

3.3.1 Analysis of Protein Deposition Patterns in the Leaf Tissue

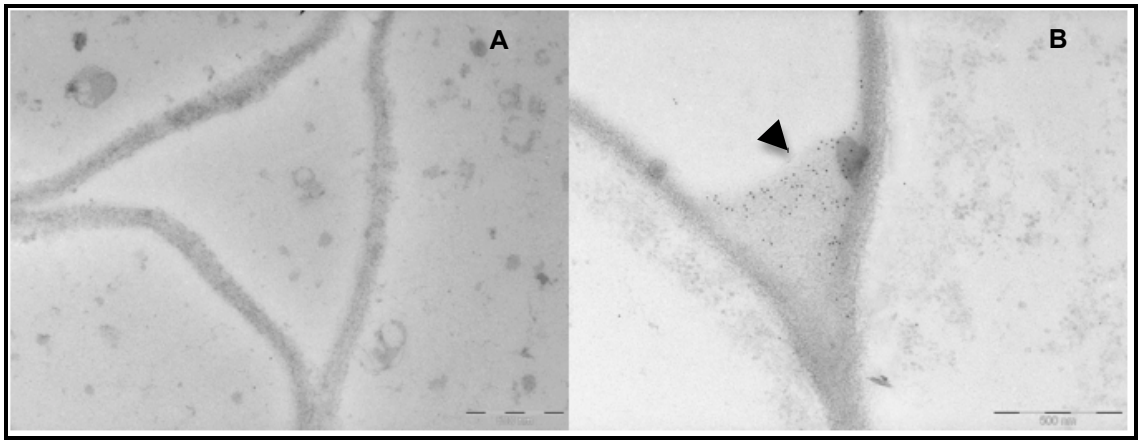

Figure 3.3.1.1 – Analysis of Protein Deposition Patterns in the Leaf Tissue. Wild type leaf (A) and transformed leaf (B) observed under the electron microscope labeled with anti – L-PGDS primary antibody and secondary antibody with 10nm gold particles. Scale bars = 500 nm. (See Appendix II for detail)

The images above were obtained as described in the material and methods chapter 2.4.

By the analysis of these pictures it is possible to see that the wild type is clear of any gold particle signal and that the L-PGDS protein is highly accumulated in the apoplast of the transformed tissue. When comparing these images with the images obtained with the fluorescence microscopy it is possible to observe that the deposition pattern is the same, and the microscopy techniques complement each other. The use of these two techniques allowed the confirmation that the L-PGDS protein accumulated in the leaf apoplast, as expected.

3.3.2 Analysis of Protein Deposition Patterns in the Root Tissue

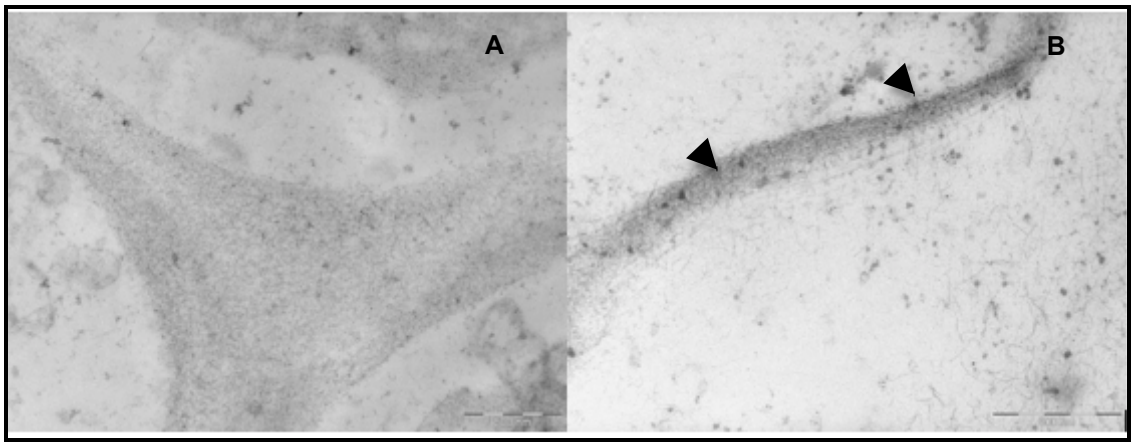

Figure 3.3.2.1 – Analysis of Protein Deposition Patterns in the Root Tissue. Wild type root (A) and transformed root (B) observed under the electron microscope labeled with anti – L-PGDS primary antibody and secondary antibody with 10nm gold particles. Scale bars = 500 nm. (See Appendix II for better detail)

The images above were obtained as described in materials and methods section 2.4.

By the analysis of these pictures it is possible to see that the wild-type root is clear of any gold particle signal and that the L-PGDS protein is accumulating near the cell wall. The paraformaldehyde fixation is not the best fixative to ensure the original conditions of the cells, and thus it is not possible to observe the apoplast in these samples. Therefore, when these images are compared with the fluorescence microscopy ones, we can suggest that the protein is being accumulated in cell wall linked structures, or even in the apoplast even though this structure cannot be seen in these images. However, the suspicion of protein accumulation within small vesicles as seen under the fluorescence microscope was abandoned since no such structures were observed.

3.4 Electron Microscopy analysis of *Medicago truncatula* Tissue Structure and Recombinant Protein Deposition Pattern

3.4.1 Analysis of Tissue Structure of Transformed and Wild-type Leaf

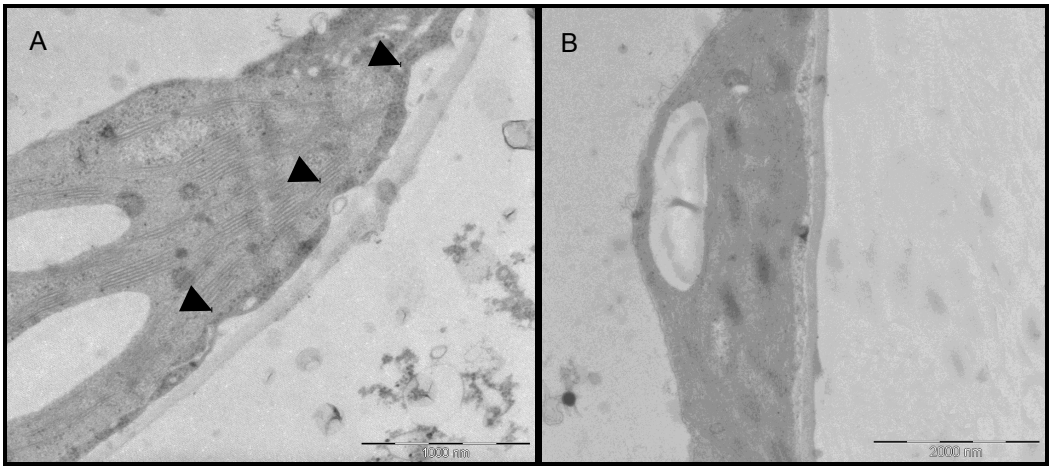

Figure 3.4.1.1 – Structure of *Medicago* leaf Tissue. Transformed leaf (A) and wild-type leaf (B) in epon resin. Scale bars: A and B = 2000 nm. Arrow heads indicating cytoplasm unattachments from the cell wall.

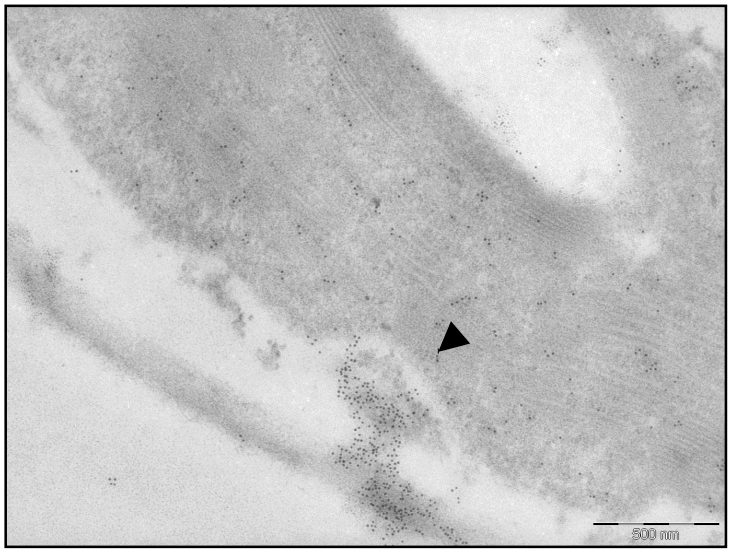

Figure 3.4.1.2 – Observation of Protein Deposition Pattern in Cellular Structure Studies. Transformed leaf in lowicryl resin labeled with anti-L-PGDS primary antibody and a secondary antibody anti-rabbit coupled to 10nm gold particles. Scale bar = 500 nm. Arrow head indicating cytoplasm unattachments from the cell wall.

The images above were obtained as described in the material and methods chapter 2.4 and 2.5.

The fixation and consequent visualization of tissue cells embedded in epon resin is important in order to analyze the structure of the cells and, possibly, to notice differences in the structure between the wild-type and the transformed cells. These differences can then be compared with the samples embedded in lowicryl resin and, possibly, the differences observed can be matched with the localization of the gold particles. The epon resin allows this type of study because the tissue fixation and embedding preserves the tissue much better than lowicryl resin.

When analyzing images A and B (figure 3.4.1), a difference in structure between transformed and wild-type cells can be observed. In the transformed cell it is possible to see that there are parts of the cytoplasm unattached from the cell wall in a way that seems not to be arbitrary. This is confirmed when analyzing the wild-type cell, in which the cytoplasm and the cloroplast seem to be normally attached to the cell wall. Several samples of these two types of cells were visualized under the electron microscope and this difference in structure was consistently observed in all of them.

When analysizing the figure 3.4.2, corresponding to a transformed cell of a tissue embedded in lowicryl resin and labeled with anti-L-PGDS primary antibody, it is worth noticing that, besides the detachment of the cytoplasm and cloroplast from the cell wall due to fixation, a protuberance in the cytoplasm and cloroplast is clear. Here, it is also possible to see a great quantity of gold particles accumulated in this area, suggesting that these detachments can be formed or caused by the accumulation of the recombinant protein L-PGDS.

In order to confirm this hypothesis, it would be helpful to repeat the process, this time optimizing the tissue fixation, to be sure that these detachments are not due to poor fixation or poor embedding process.

3.4.2 Subcellular Deposition Analysis of L-PGDS by Electron Microscopy (continuation of chapter 3.3)

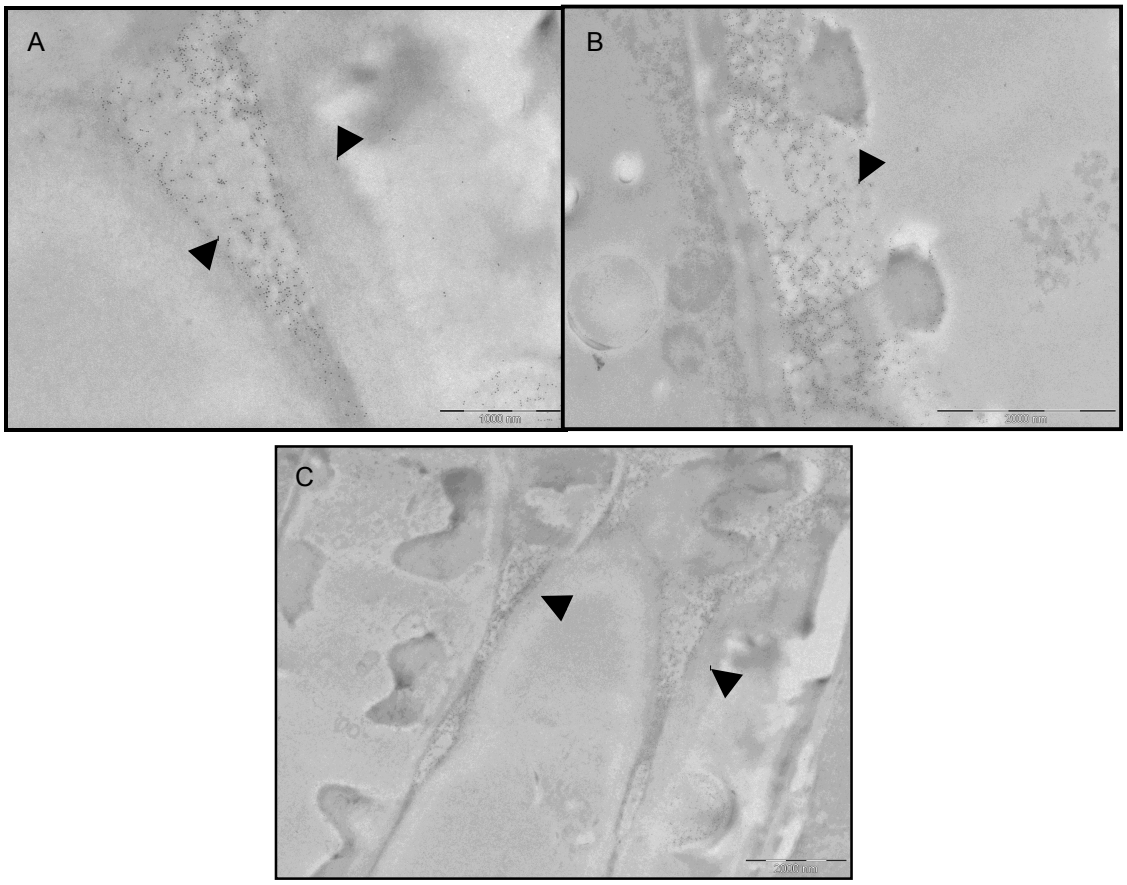

Figure 3.4.2.1 – Analysis of Protein Deposition Pattern in the Leaf Tissue. Transformed leaf labeled with anti-L-PGDS primary antibody,secondary antibody is an anti-rabbit coupled with 10nm gold particles. Scale bars: A =1000 nm, B and C = 2000 nm. Arrow heads indicating recombinant gold particles deposition.

The images above were obtained as described in the material and methods chapter 2.4.

During my second stay in Valencia, a different kind of fixation was tested. The tissues were fixed using a 4% paraformaldehyde solution and, instead of incubating the tissues in this solution directly at 4ºC overnight, the incubation started at room temperature for 5 hours and then overnight at 4ºC. The results achieved with this change in the fixation procedure showed to improve the integrity of the cell components. The improvement of the tissue quality seems to be crucial to the visualization of the gold particles. Labeling was performed using anti-L-PGDS primary antibody (1:100) and a secondary anti-rabbit antibody with 10 nm gold particles. In figure 3.4.1.1 there is a considerable amount of gold particles located near the xylem. The gold particles accumulate in the intercellular spaces as also as between the cell wall and the tracheids.

This unexpected subcellular localization might be due to several factors. One is that if the recombinant protein is detected in the leaf xylem, it could possibly be transported from the root to the rest of the plant, since this kind of transport is the major function of the vessel tissue. The presence of the L-PGDS protein in the root tissue is verified by the results of both western blot and fluorescence microscopy. Another fact worth to be noticed is that the tracheids are known to be conductive cells, which might explain the presence of gold labeling around these structures. Furthermore, xylem parenchyma cells can be modified to become transfer cells (characteristic of phloem), a type of cells which presents cell wall ingrowths that increase the surface area of the plasma membrane, thus increasing the solute transfer across the membrane (Taiz & Zeiger, 1991). This type of cells probably serves to retrieve or/and reroute solutes that move through the xylem. So, the recombinant protein can be somehow accumulated in this site to be redirected to another location (intracellular spaces, apoplast). Another possible explanation is the function of the phloem companion cells. These cells are thought to take over some metabolic functions like the protein synthesis, since this function is reduced or lost during the differentiation of the sieve elements (Taiz & Zeiger, 1991). Thus, companion cells may be producing the recombinant L-PGDS and, once xylem and phloem are very close together, the protein may be accumulating near these tissues for this reason (Taiz & Zeiger,1991).

To better understand better this issue, it would be useful to use electron microcopy to analyze the subcellular localization of the recombinant L-PGDS in the xylem and phloem of the root tissue.

4 Conclusions and Future Perspectives

This master thesis project aimed at studying the subcellular localization of a human protein with a complex glycosylation pattern, named Prostaglandin D_2 Synthase lipocalin type (L-PGDS), in a model plant species. This protein is the most abundant in the cerebrospinal fluid and has several important roles in physiological and pathological human functions, so its absence can result in several diseases.

Medicago truncatula, the model plant species for the legume family used in this study, is easy to transform and manipulate in the lab and its genome is totally sequenced. Thus, it is being used in the lab for studies involving Molecular Farming. Since Molecular Farming seems a good alternative to the systems used nowadays and this protein production is important in terms of therapeutics, the study of its expression in plants seems an interesting challenge.

Therefore, the work developed in this thesis is a novelty since the expression of L-PGDS in a plant tissue or cell has never been reported. To perform this study, plants and suspension cultures expressing L-PGDS in the secreted form were used. Leaves, roots, seeds of *M. truncatula* and liquid medium from the *M. truncatula* suspension cell cultures were analyzed by SDS-PAGE electrophoresis in order to determine the presence of the protein. Roots and seeds expressed L-PGDS with a molecular weight of 26 kDa, as expected for this protein. In leaves and cell medium a range of molecular weights were detected, 26-29 kDa and 28,5-36 kDa, respectively. This can be due to the presence of more than one glycoform of the protein in these cells. Nevertheless, the signal obtained in the blotting membrane for the cell medium was very inconsistent throughout extractions obtained at different time points. Thus, the level of protein expression and the growth of the cell cultures have first to stabilize for a more conclusive study to be carried out.

When comparing the results obtained by SDS-PAGE electrophoresis and Western Blotting, the presence of only one glycoform in roots and seeds would be an advantage when compared to leaves and cell medium, once protein homogeneity is one of the principal requirements for its approval for therapeutic applications.

The study of the protein trafficking and subcellular localization is important to determine if it is possible to obtain functional and non-imunogenic proteins and it is also crucial for economical reasons since the location where the protein is accumulating within the cell will dictate the downstream processes needed for obtaining the recombinant protein. The secreted form of recombinant proteins seems to be the more viable economically, both in plant systems and in cell cultures, since there is no need to proceed to a total disruption of the tissue or the cell.

In this work, immunolocalization studies of the secreted form of L-PGDS, expressed in the whole plant system and in the suspension cell cultures, were performed using fluorescence and electron microscopy.

In the fluorescence microscopy studies, L-PGDS was found accumulating in the apoplast and in structures linked to the cell wall of leaves and roots; in the roots it seemed that the

protein was aggregated in small vesicles. In the seeds, the pattern was not obvious but it tended to accumulate in the apoplast and within the cell. In the suspension cells the recombinant protein deposition pattern was not clear but it appears to accumulate within the cell, maybe in small vesicles, until it is secreted to the cell medium. Future work with the fluorescence microscopy technique would involve a more detailed study of the protein accumulation by simultaneously dyeing the ER or the Golgi apparatus.

In the electron microscopy studies, L-PGDS deposition patterns were analyzed in leaves and roots. Seeds were not analyzed since they were not developed by the time I traveled to Valencia to process the samples for electron microscopy. The recombinant protein was found accumulating in the apoplast of leaf and root cells. Using this technique it was possible to confirm the results obtained by fluorescence microscopy and to exclude the possibility that L-PGDS was accumulating in small vesicles in the roots, since no alike structures were detected. However, future work must be done in order to obtain a more significant protein signal. To do so, a more specific primary antibody has to be applied, since the one used is not sufficiently specific for the detail of the electron microscopy. In addition, the optimization of fixation and embedding processes have also to be done, since chemical fixation resulted in loss of tissue characteristics and a better embedding would improve the tissue section integrity.

Hence, this project needs future work, due to its duration and to the fact that is innovative and, thus, the results obtained are preliminary.

Although plants present several advantages for the production of recombinant proteins, as mentioned in the introduction chapter, one of the reasons for lack of investment is the incapacity of totally reproducing mammalian N-glycosylation. Glycoproteins produced in plants do not have β(1,4) galactose and sialic acid, and present two residues that do not exist in mammalian proteins, β(1,2) xylose and α(1,3) fucose. The management of these residues is very important for the protein functionality, so it is critical to modify the N-glycosylation pathway in a way that eliminates the immunogenicity caused by non-mammalian residues.

Since this protein is a secreted form, it should contain N-glycans of the complex type (containing immunogenic residues like α (1,2) fucose and β (1,3) xylose), it is necessary to purify the protein to assess, by mass spectrometry, which glycoforms are present in each tissue as well as understanding which glycosylation patterns are present. With this information it will be possible to optimize the N-glycan humanization strategies, for example to include the addition of sialic acid linked to a galactose residue as described in the introduction section 1.6.1. Besides that, N-glycosylation pathway in *Medicago truncatula* has also to be further investigated so changes in the pathway can be made to adapt it to the transformations needed in the protein. Thus, each case is unique since the characteristics of the final protein will depend on the protein and on the plant that it is being produced, in which tissue it accumulates and even in what compartment of the plant cell (Abranches *et al.*, 2008; Drakakaki *et al.*, 2006).

Future work would also involve analyzing the differences in the structure, biochemical properties and stability of the final product when compared with the native form of the protein. It

would also be important to analyze the enzyme activity, assessing its activity by testing the substrate conversion rate *in vivo*.

The results presented in this work showed that whole-plant systems and suspensions cell cultures of *Medicago truncatula* are promising as a Molecular Farming platform, although some optimizations are needed. When comparing whole plant system with plant cell cultures for the production of L-PGDS, each has its own advantages and disadvantages, as discussed in the introduction. Regarding the production in the different organs of the plant the fluorescence signal visualized by microscopy analysis was consistent with the western blotting analysis while fluorescence signal of cultured cells was not conclusive with the western blotting analysis, once it is very difficult to detect a protein signal without sample concentration. In addition, the protein under study accumulates mainly in the apoplast and cell wall (leaf and root), being easier to extract the protein for purification if needed than when it accumulates inside the cell.

Despite the fact that obtaining the protein is easier in a cell culture suspension system, since the protein is secreted and it is only necessary to process the cell medium, the whole-plant system seems to be more advantageous once it suggests that the protein production is higher.

This master thesis can fit as groundwork for future investigations in this field, in order to establish effective plant systems for Molecular Farming.

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6 Appendix

6.1 Reagents and Solutions

Fixation Solution:

4% of Paraformaldehyde (Merck, USA) in MTSB

4g of paraformaldehyde are added to 50 mL distilled water. The solution is heated at 60ºC for 15 minutes on a heat-stir (heat-stir SB162, Stuart, United Kingdom), stirring, and 1 drop of NaOH (Panreac, Spain) 1M is added. Then the solution was left to cool down in a hotte and 50 mL 2X MTSB was added. The solution is unstable so it has to be prepared just before use.

Stacking Gel Buffer Stock:

0.5 M Tris-HCl (pH 6.8)

Tris – Base (Promega, USA) is dissolved in distilled water. Then, the pH is adjusted with HCl 1M in a pH meter (pH meter Basic 20, Crison, Spain) to 6.8 and distilled water is added to the final volume. The solution is stored at 4ºC

Resolving Buffer Stock:

3 M Tris-HCl (pH 8.8)

Tris – Base is dissolved in HCl 1M. Distilled water is added to the final volume and pH adjusted. The solution is stored at 4ºC.

Running Buffer Stock (10x):

25 mM Tris-Base
192 mM Glycine
0,1% SDS, pH 8.3

Tris – Base, glycine are dissolved in distilled water and SDS is added. pH is adjusted to 8.3 with HCl 1M and the solution is stored at 4ºC.

10% SDS (Sodium Dodecyl Sulphate):

10 g of SDS are dissolved in 1L of distilled water. Heat up to dissolve. The solution is stored at room temperature.

1.5% Ammonium Persulphate:

0.15 g of ammonium persulphate is dissolved in 10 mL of distilled water. This solution is unstable so it should be made just before using it.

Protein Extraction Buffer:

100 mM of Ascorbic Acid
500 mM of NaCl
5mM of β-mercaptoethanol

Ascorbic acid and NaCl are dissolved in distilled water and β-mercaptoethanol is added. pH is adjusted to 8.0 to NaOH 1M. The solution is stored at 4ºC.

Sample Buffer 4x:

0,08 M Tris-HCl pH 6.8
2% SDS
5% β-mercaptoethanol
10% Glycerol
0.001% Bromophenol Blue

The final volume is completed with distilled water. The solution is stored at -20ºC.

Murashing & Skoog (MS) Medium 20 x:

215.1 g of MS are diluted in 5 L of distilled water. Then, the solution is transferred to 50 mL tubes and stored at -20ºC.

Culture Medium MS Agar:

30 g of Saccharose
50 mL of Stock Solution MS 20x concentrated
7g of Agar (Duchefa Biochemie, The Netherlands)

Saccharose is dissolved in distilled water and the MS 20x is added and the solution is transferred to Schott bottles (Schott, USA) with the Agar weighted previously, being autoclaved afterwards. The solution is stored at room temperature.

Protein Transfer Buffer:

20% Methanol
10% Running Buffer (10x concentrated)

Running buffer and methanol are diluted in distilled water. The solution is stored at room temperature.

Phosphate buffered saline (PBS) 10x:

1.36 M of NaCl
26,9 mM of KCl
16,8 mM of Na_2HPO_4
15 mM KH_2PO_4

The ingredients are dissolved in distilled water and pH adjusted to 7,6. The solution is stored at room temperature.

PBS 1x: 0,1% of PBS 10x is diluted in distilled water.

PBS-T: 0,1% of Tween® 20 (Sigma – Aldrich, USA) is diluted in PBS 1x.

MTSB 2x:

50mM PIPES
5mM $MgSO_4$
5mM EGTA

The solution is prepared in distilled water. NaOH is added to help dissolve the EGTA (is insoluble in acidic solution) and then pH is adjusted to 6.9 with dissolved H_2SO_4. The solution is stored at room temperature.

Tris Buffered Saline Buffer (TBS) 10x:

1 M Tris-HCl pH 7.4
5 M NaCl

The ingredients are diluted in distilled water. The solution is stored at room temperature.

TBS 1x: TBS 10x is diluted in distilled water.

2.5% Agar:

2.5 g of agar are dissolved in 1 L of distilled water. The solution is heated up until total dissolution of agar. The solution is stored at room temperature.

Coomassie Brilliant Blue G-250:

45% Methanol (100%)
10% Glacial Acetic Acid
0,1% Commassie Blue

The solution is stored at room temperature.

Coomassie Dye De-staining Solution:

45% methanol
10% Glacial Acetic Acid

Methanol and glacial acetic acid are added to distilled water. The solution is stored at room temperature.

NBT/BCIP Buffer:

0.1 M of Tris-HCl pH 9.0
0.01 M of NaCl
0.05 M $MgCl_2$

The ingredients are dissolved in distilled water. The solution is stored at 4ºC.

NBT/BCIP Detection Solution:

6.6 μL of BCIP (Promega, USA)
13.2 μL of NBT (Promega, USA)

BCIP and NBT are added to NBT/BCIP Buffer, in an eppendorf covered with aluminium paper. This solution is prepared just before use.

DAPI:

DAPI stock solution (1.6 mg/mL) is diluted in distilled water to obtain the final solution (1μg/mL).

Lowicryl Resin:

To prepare 100 g of Lowicryl resin:
13.5 g of Crosslinker A (Electron Microscopy Sciences, USA)
86.5 g of Monomer B (Electron Microscopy Sciences, USA)
0.50 g of Initiator (Electron Microscopy Sciences, USA).

Stir and store at 4ºC.

Uranyl Acetate Solution:

0,5% uranyl acetate (Electron Microscopy Sciences, USA) are diluted in 100% methanol (Panreac, Spain).

Lead Cytrate Solution (Reynolds Solution):

Solution A: Lead Nitrate 1M
Solution B: Trisodic Cytrate 1M
Solution C: NaOH 1M

Distilled water is boiled to eliminate carbon dioxide, since it precipitates lead, and placed in a 50 mL tube. Then, the tube is placed under cool running water. Finally, NaOH is added.

The final solution is prepared in a 50 mL tube adding 16 mL of distilled water and 3 mL of solution B, stirring slowly by inversion. Then, 2 mL of solution A are added, stirring until the precipitate formed is totally dissolved. 4 mL of solution C are added slowly until the solution turns transparent, stirring by inversion ocasionally. The final solution is filtered with a syringe

with a filter (Whatman 0.2 μm PTFE w/GMF) to the immunodetection parafilm. The solution can be maintained at room temperature, in a vertical position.

0,025 M Cacodylate Solution:

0,025 M cacodylic acid (Electron Microscopy Sciences, USA) are added to distilled water and dissolved by stirring.
pH is adjusted to 6.9 and then the solution is transferred to an 500 mL Schott bottle and stored at 4ºC.

Karnosvky Fixative Solution:

16% Paraformaldehyde (Electron Microscopy Sciences, USA)
25% Glutaraldehyde (Electron Microscopy Sciences, USA)
0.006M Calcium Chloride

The ingredients are diluted in 0.025 M cacodylate solution. Paraformaldehyde is added as also as the glutaraldehyde. The calcium chloride is weighted in a different tube and diluted in cacodylate solution, adjusting the pH to 6.9. Then, calcium chloride solution is added to the paraformaldehyde and glutaraldehyde solution and stirred by inversion. The final solution is divided for several eppedorfs and stored at -20ºC.

Osmium Solution:

0.033 M Cacodylate Solution
4% OsO_4 (Electron Microscopy Sciences, USA)

The final solution is divided into several eppendorfs covered with aluminium foil paper and placed in liquid nitrogen. When frozen, eppendorfs were stored at -20ºC.

Epon Resin Preparation:

Epon resin is an aliphatic hydrophobic epoxi resin. In a hotte, two solutions are prepared as follows.
Solution A: 2.2 M DDSA (Electron Microscopy Sciences, USA) and 4.5 g of Epon 812-substitute (Electron Microscopy Sciences, USA). The solution is stirred until it became homogeneous.
Solution B: 2.6 M NMA (Electron Microscopy Sciences, USA) and 5.5 g of Epon 812-substitute. The solution is stirred until it became homogeneous.

Then, the two solutions are mixed and 0.10 M BDMA is added, since it is an accelerator, favouring the polymerization. The solution is stirred slowly for 20 minutes, to avoid the formation of bubbles. The solution is stored in a falcon tube at 4ºC.

6.2 Electron Microscopy Images

Wild-type and Transgenic Leaves

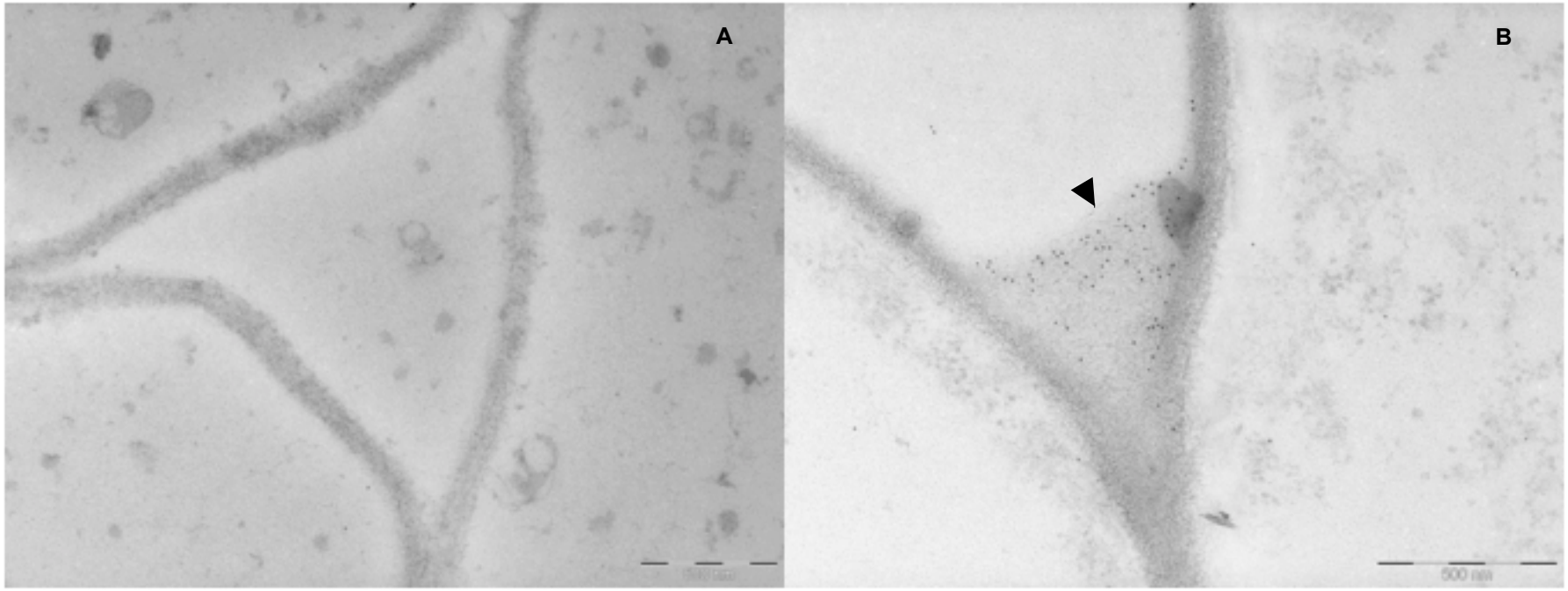

Figure 6.2.1 – Analysis of Protein Deposition in the Leaf Tissue. Wild type leaf (A) and transgenic leaf (B) observed under the electron microscope. It is noticeable the considerable accumulation of gold particles in the cell apoplast (B). Scale bars: 500 nm.

Wild-type and Transgenic Roots

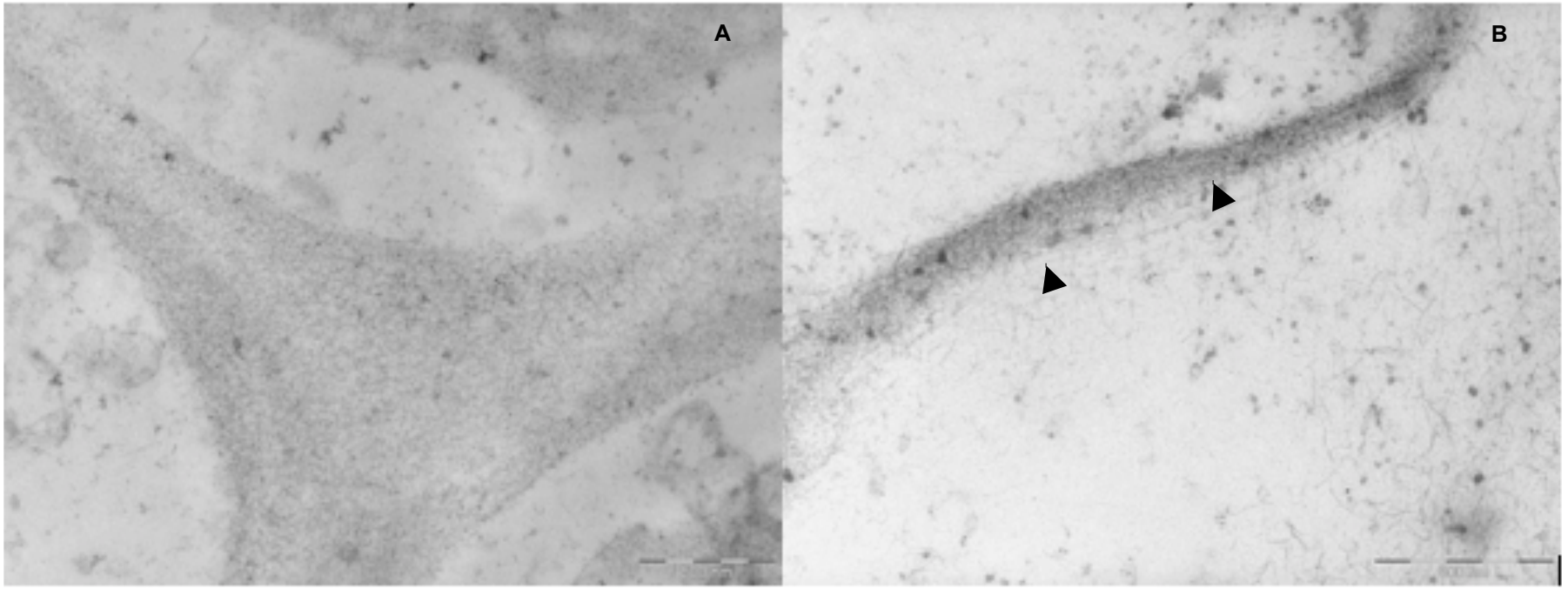

Figure 6.2.2 – Analysis of Protein Deposition Patterns in the Root Tissue. Wild type root (A) and transgenic root (B) observed under the electron microscope Note the gold particles accumulating in the cell wall (B). Scale bars: 500 nm.

6.3 Work Presented in Congresses during the Master Thesis Development

The following abstract was presented, as a poster, in the Interbio Symposium "Frontiers in Protein Research", held in Oeiras, 5th to 7th May 2011.

Sorting of recombinant proteins in the plant secretory pathway

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Abstract:

Molecular farming – the production of recombinant proteins in plants – is a promising alternative to microbial or animal systems for the production of high value molecules. Plant-produced molecules present significant reduced costs and are mainly applied to therapeutics, diagnostics and industrial enzymes.

We have been using the legume plant *Medicago truncatula* for the production of recombinant proteins which are targeted to the secretory pathway and are either secreted or retained intracellularly. In this work, we investigated the deposition patterns of a human protein, prostaglandin D_2 synthase lipocalin type or β-trace, in different organs of *Medicago* plants, to address the tissue specific factors that affect the successful production and accumulation of recombinant proteins. By using immunolabeling and fluorescence microscopy, we observed significant differences in subcellular deposition of the recombinant protein in leaves, seeds and roots, which affected glycosylation profiles and consequently the properties of the purified product. Understanding the plant secretory pathway is thus essential for assessing and optimizing plant systems for the production of human therapeutics.

Preliminary results show different deposition patterns in leaf, root and seed, to be confirmed by western blotting and fluorescence microscopy.

The following abstract was presented, as an oral presentation, in the Microscopy at the Frontiers of Science Congress, held in Aveiro, 18th to 21st October 2011.

Microscopy analysis of recombinant protein deposition patterns in transgenic *Medicago* plants

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Abstract:

Molecular Farming – the production of recombinant proteins in plants – is a promising alternative to microbial or animal systems for the production of high value molecules [1]. Plant-produced molecules present significant reduced costs and are mainly applied to therapeutics, diagnostics and industrial enzymes. Understanding the different pathways that a protein can take in a plant tissue is essential for assessing and optimizing plant systems for the production of human therapeutics. Microscopy is one of the most important techniques to achieve this knowledge, since it reveals the localization of the recombinant proteins within the plant cell, thus allowing to assess possibly different protein glycoforms that may be produced. This information is crucial to understand if the recombinant protein is suitable for human therapeutics, and also to devise the best procedures for purification of the recombinant product.

We have been using the legume plant *Medicago truncatula* for the production of recombinant proteins [2], which are targeted to the secretory pathway and are either secreted or retained intracellularly. In this work, we investigated the deposition patterns of a human protein, prostaglandin D2 synthase lipocalin type or β - trace, in different organs of *Medicago* plants, to address the tissue specific factors that affect the successful production and accumulation of recombinant proteins. By using immunolabeling and fluorescence microscopy, we observed significant differences in subcellular deposition of the recombinant protein in leaves, seeds and roots, which affected glycosylation profiles and consequently the properties of the purified product. We demonstrate that microscopy is a crucial method for Molecular Farming to progress.

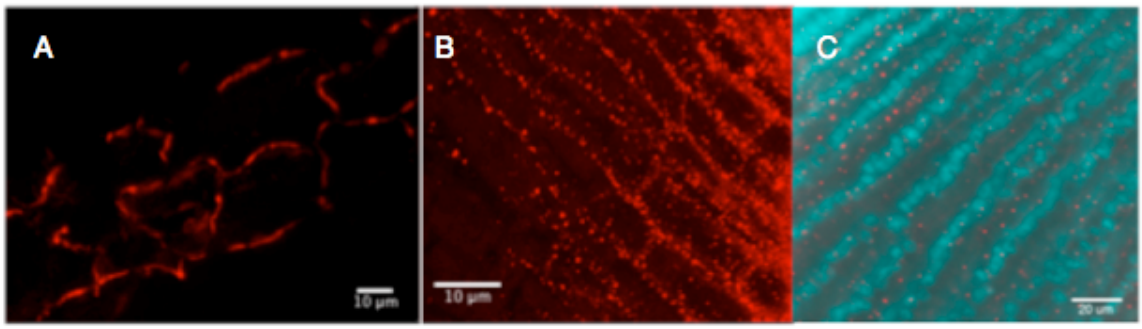

Figure 6.3.1 – Localization of the recombinant protein in transgenic *Medicago truncatula* plants by fluorescence microscopy. (A) leaf, (B) root, (C) seed. Blue – DAPI; Red – specific antibody for L-PGDS.

[1] Fischer, R., Emans, N., Transgenic research, 9 (2000) 279-299.

[2] Abranches *et al.* Journal of Biotechnology, 120 (2005) 121-134.